Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen

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Abstract

Traditional methods for enumerating and identifying microbial populations within the rumen can be time consuming and cumbersome. Methods that involve culturing and microscopy can also be inconclusive, particularly when studying anaerobic rumen fungi. A real-time PCR SYBR Green assay, using PCR primers to target total rumen fungi and the cellulolytic bacteria *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*, is described, including design and validation. The DNA and crude protein contents with respect to the fungal biomass of both polycentric and monocentric fungal isolates were investigated across the fungal growth stages to aid in standard curve generation. The primer sets used were found to be target specific with no detectable cross-reactivity. Subsequently, the real-time PCR assay was employed in a study to detect these populations within cattle rumen. The anaerobic fungal target was observed to increase 3.6-fold from 0 to 12 h after feeding. The results also indicated a 5.4-fold increase in *F. succinogenes* target between 0 and 12 h after feeding, whereas *R. flavefaciens* was observed to maintain more or less consistent levels. This is the first report of a real-time PCR assay to estimate the rumen anaerobic fungal population.

Introduction

The digestion of plant material and subsequent conversion for energy requirements to the host ruminant are performed through a complex symbiotic relationship of microbiota within the rumen (Mackie, 1997). The composition and proportion of microorganisms are influenced by external factors, such as diet, feeding frequency, age, geographical location and ruminant–host interaction (Hungate, 1966; Dehority & Orpin, 1997). A need to better understand the intricacies of these interactions first led researchers to investigate what were considered to be key microbial members within the rumen with respect to fatty acid metabolism, fibre degradation, methanogenesis and bacterial predation (Mackie, 1997). Much research has focused on the cellulolytic degrading microorganisms and the effect of diet and feeding frequency on these populations (Krause *et al*., 2003).

Early studies involved direct counting of bacteria using traditional enumeration methods such as microscopy and colony counts (Leedle *et al*., 1982). Most research has centred on the role of the three prominent fibre-degrading bacteria *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. In addition to the major cellulolytic bacterial populations, the rumen also possesses highly fibrolytic anaerobic rumen fungi. Anaerobic rumen fungi were first described by Orpin (1975) and are thought to play an important role in fibre degradation within the rumen (Orpin & Joblin, 1997).

Anaerobic filamentous fungal populations have proven to be more difficult to enumerate than bacteria, mainly as a result of their dual life stages: a motile free-swimming zoospore and then a fibre-attached mature thallus (Orpin, 1994). Initially, enumeration of anaerobic fungi focused on zoospore counts (Orpin, 1975). Zoospore counts could not be completely extrapolated to thallus-forming units (TFUs), particularly when considering polycentric species, which are capable of forming new rhizobium from fragments of old rhizomycelium in addition to zoospores (Hespell *et al*., 1997). A most probable number (MPN) method of serially diluted fungal samples was developed to calculate TFUs (Theodorou *et al*., 1990; Obispo & Dehority, 1992). This method revealed that zoospore counts, although much more rapid, were underestimating the fungal populations of the rumen.

With the advancement of molecular enumeration methods, in particular 16S/18S rRNA gene probing methods, researchers were able to monitor bacterial and fungal species within the rumen (Stahl *et al*., 1988; Dore *et al*., 1993).

Because of the high level of conservation within the fungal 18S rDNA gene sequence (Bowman *et al*., 1992), a more appropriate target for identification, the internal transcribed spacer 1 region (ITS1), should be targeted. Located between the 18S rDNA and 5.8S rDNA genes, this region was identified as containing high levels of sequence variation, and is used for the phylogenetic identification of anaerobic rumen fungi (Li & Heath, 1992; Brookman *et al*., 2000).

Real-time PCR is a powerful tool that allows for the rapid quantification (Freeman *et al*., 1999) of a target DNA sequence through the design of specific primer sets. Researchers have shown that this technique can be used successfully on samples extracted from rumen contents to monitor populations in the rumen (Tajima *et al*., 2001; Ouwerkerk *et al*., 2002; Klieve *et al*., 2003). In this study, we describe the design and use of a SYBR Green real-time PCR assay to monitor two fibrolytic bacterial species, *F. succinogenes* and *R. flavefaciens*, and the total anaerobic fungal population. A growth curve study of anaerobic fungal isolates, in terms of their DNA and protein contents with respect to fungal biomass at various stages, was performed. This allowed for the generation of standard curves based on fungal DNA from pure cultures, thus enabling the calculation of fungal biomass or fungal contribution to microbial nitrogen within the rumen. This is the first description of a real-time PCR assay that can rapidly monitor anaerobic rumen fungal populations.

Materials and methods

Bacterial cultures and growth medium

All anaerobic microbial cultures were grown on media 10 plus 30% rumen fluid under anaerobic conditions at 39 °C (Caldwell & Bryant, 1966). The following strains of rumen bacteria were used as reference strains: *Fibrobacter succinogenes* (S85, BL2, HM2 and REH9-1) (gift from Professor R. Mackie, University of Illinois, IL), *Ruminococcus albus* (AR67) and *Ruminococcus flavefaciens* (Y1 and R13e2) (Krause *et al*., 1999). Additional rumen bacterial strains, as indicated in Table 1, were taken from our existing collection. Several anaerobic fungal isolates from our collection were also used for reference purposes when developing these methods: *Neocallimastix* spp. isolates CX, LM1 and PN1 from sheep (McSweeney *et al*., 1994) and TBT2 from banteng, *Orpinomyces* spp. isolates F9 and F11 from banteng and *Piromyces* spp. isolates TNL1 from nilgai, TZB2 from zebra and KS13 from kangaroo (Tuckwell *et al*., 2005). Nine aerobic chytrid fungal isolates covering three of the four aerobic orders were grown on PYG 2% agar at 22 °C (gift from Drs F. Gleason and P. McGee, University of Sydney, Sydney, Australia). The aerobic fungal isolates were as follows: order *Blasticladiales*, one isolate from the family *Blastocladiacea* (*Allomyces arbuscula* Allo Mar CW16) and two from the family *Catenariacea* (*Catenaria* sp. Poly AD-20 and *Catenophlyctis* sp. CC4-10Z); two fungal isolates from the family *Chytridiacea* within the order *Chytridiales* (*Cladochytrium* sp. AUS11 and *Rhizophydium* sp. AUS7); and, finally, within the order *Spizellomycetates*, three isolates from the family *Spizellomycetaceae* (*Rhyizophlyctis* sp. AUS16, *Rhyizophlyctis rosa* AUS13 and *Spizellomyces* sp. Mar Ad 2-0) and one isolate from the family *Olipidiaceae* (*Powellomyces* sp. AUS16).

Fungal growth curves

Two monocentric fungi, *Neocallimastix* sp. CX and *Piromyces* sp. TGB1, and a polycentric isolate, *Orpinomyces* sp. F11, were inoculated individually into six 10-mL anaerobic culture media tubes and grown at 39 °C. At 6-h time intervals, three tubes for each fungus were harvested by centrifugation at 10 000 ***g*** for 10 min. The fungal cell pellet was transferred to a preweighed tube and freeze dried for 24 h. The dried fungal biomass was then weighed to determine the total amount of dry weight fungal biomass at each time point.

A quantity of known biomass from each time point was then used to determine the percentage nitrogen. A weighed aliquot was placed into a sample boat and combusted at 1100 °C using a LECO CNS-2000 Combustion Analyser. Total nitrogen was measured using a thermal conductivity detector with EDTA as a calibration standard (Analytical Services, School of Land and Food, The University of Queensland, Qld., Australia). Crude protein values were calculated by multiplying the nitrogen value by 6.25.

DNA was extracted at each time point from a known quantity of dry weight fungal biomass using the methods described below.

Rumen sampling

Samples of rumen fluid and digesta were extracted from four fistulated Brahman crossbred steers [212 ± 4.2 kg liveweight (mean $\pm$ SEM)] which were fed a basal diet of Angleton grass (*Dicanthium aristatum*) which was deficient in sulphur, nitrogen and phosphorus. Urea/phosphoric acid supplement solution (urea, 447.4 g L^{-1}; 85% w/w orthophosphoric acid, 98.5 g L^{-1}) was sprinkled on the feed (34.4 mL kg^{-1}) to increase the nitrogen and phosphorus content to 1.2% and 0.27%, respectively, on a dry weight basis. The nitrogen to sulphur ratio in the feed was 0.055 and was considered to be sulphur deficient. Digesta samples were directly taken from three sites within the central portion of the rumen (anterior, central and posterior) with initial course filtration through an insect screen with a medium mesh size (2×1.5 mm). The pooled filtrate, in excess of 500 mL per sampling, contained digesta plant

Table 1. PCR primer validation with various microbial DNA templates

Template DNA	Bacterial	Fungal	*Ruminococcus flavefaciens*	*Fibrobacter succinogenes*
Neocallimastix sp. CX	–	+	–	–
Neocallimastix sp. LM1	–	+	–	–
Neocallimastix sp. PN1	–	+	–	–
Neocallimastix sp. TBT2	–	+	–	–
Orpinomyces sp. F9	–	+	–	–
Orpinomyces sp. F11	–	+	–	–
Piromyces sp. KS13	–	+	–	–
Piromyces sp. TGB1	–	+	–	–
Piromyces sp. TNL1	–	+	–	–
Allomyces arbuscula Allo Mar CW16	–	–	–	–
Catenaria sp. Poly Ad-20	–	–	–	–
Catenophlyctis sp. CC 4-10Z	–	–	–	–
Cladochytrium sp. AUS11	–	–	–	–
Powellomyces sp. AUS17	–	–	–	–
Rhyizophlyctis sp. AUS16	–	–	–	–
Rhyizophlyctis rosea AUS13	–	–	–	–
Rhizophydium sp. AUS7	–	–	–	–
Spizellomyces sp. Mar Ad 2-0	–	–	–	–
Saccharomyces cerevisiae	–	–	–	–
Butyrivibrio fibrisolvens (AcTF2)	+	–	–	–
Escherichia coli K12	+	–	–	–
Prevotella ruminocola (23)	+	–	–	–
Ruminococcus albus (AR67)	+	–	–	–
Selenomonas ruminantium (AR56)	+	–	–	–
Streptococcus bovis	+	–	–	–
Ruminococcus flavefaciens (R13e2)	+	–	+	–
Ruminococcus flavefaciens (Y1)	+	–	+	–
Fibrobacter succinogenes (S85)	+	–	–	+
Fibrobacter succinogenes (BL2)	+	–	–	+
Fibrobacter succinogenes (HM2)	+	–	–	+
Fibrobacter succinogenes (REH9-1)	+	–	–	+
Rumen	+	+	+	+
Soil	+	–	ND	ND
Grass	+	–	ND	ND

particles and rumen fluid, and was used for DNA extraction. Samples were taken just prior to feeding and at 4, 8, 12 and 24 h after feeding.

DNA extraction

Total genomic DNA was isolated from pure bacterial and fungal species, grass and soil samples collected from a nongrazing pasture and rumen samples using the FastDNA Kit and FastPrep Instrument (Q-BIO gene, QC, Canada). For rumen samples, a 1.5-mL aliquot was taken from the 500-mL sample using a wide-bore pipette, so as to ensure that a homogeneous sample containing plant particles and liquid was obtained. For soil and grass samples, 100 mg of material was used per extraction. For pure cultures, a known quantity of dry weight fungal biomass was used. All samples were placed in the supplied screw cap microcentrifuge tube containing irregularly shaped garnet particles and a single 0.25-in ceramic sphere. One millilitre of supplied lysis buffer was then added and the tubes were placed in the Fast Prep Instrument and processed (bead beat) at a setting of 5 for 2 min. Following this, the samples were incubated at 70 °C for 10 min and then centrifuged for 15 min at 14 000 ***g*** at 4 °C. The supernatant was transferred to a new tube where an equal volume of the supplied glass milk was added and the DNA was allowed to bind. The glass milk solution was centrifuged and then washed before the DNA was eluted with 100 μL of sterile water.

Real-time PCR primer design and assay conditions

The designed primers used for the real-time PCR are described in Table 2. The primers for detecting total bacterial 16S rRNA gene sequences were designed by firstly modifying the existing universal bacterial primer 1114f

Table 2. PCR primers for real-time PCR assay

Target species	Forward primer	Reverse primer	Size (bp)
General bacteria	CGGCAACGAGCGCAACCC* (1114)	CCATTGTAGCACGTGTGTAGCC (1275)	130
General anaerobic fungi	GAGGAAGTAAAAGTCGTAACAAGGTTTC	CAAATTCACAAAGGGTAGGATGATT	120
Fibrobacter succinogenes	GTTCGGAATTACTGGGCGTAAA (586)	CGCCTGCCCCTGAACTATC (706)	121
Ruminococcus flavefaciens	CGAACGGAGATAATTTGAGTTTACTTAGG (96)	CGGTCTCTGTATGTTATGAGGTATTACC (220)	132

*Modified from Lane (1991).

Numbers in parentheses represent *Escherichia coli* numbering positions.

(Lane, 1991) to increase the melting temperature (T_m) to 60 °C. The reverse primer was designed at a distance of 130 bp 3′ of the forward primer based on the ARB 16S ribosomal sequence multiple alignments downloaded from the ribosomal database project (RDP II). Primers were analysed using primer express (Applied Biosystems, Foster City, CA) and designed for an optimal T_m of 60 °C. Primers were then compared with sequences available at the National Center for Biotechnology Information (NCBI) via a BLAST search to ascertain primer specificity (Altschul *et al.*, 1990), and against the RDP II and ARB databases using the probe match analysis function (Cole *et al.*, 2003; Ludwig *et al.*, 2004). A similar regime was followed for the design of the two fibrolytic bacterial primer sets to target *F. succinogenes* and *R. flavefaciens* species. The general anaerobic fungal primer set was designed from multiple alignments of fungal 18S ribosomal and ITS1 gene sequences, which included all available anaerobic fungal sequences and representatives of closely and distantly related fungal species.

Conventional PCRs for the validation of the specificity of the designed primers against target genes were performed in 30-μL reactions with the addition of 2.5 mM $MgCl_2$ and employing Platinum Taq (Invitrogen, Carlsbad, CA). Reactions were performed using a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA) under the following conditions: one cycle at 94 °C for 2 min, and 40 cycles of 94 °C for 30 s, 60 °C for 15 s and 68 °C for 1 min. The PCR products were analysed by running on 2% agarose gels containing ethidium bromide and visualizing for a single specific band and the absence of primer dimer products.

Real-time PCR assays were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Assays were set up using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Optimization of assay conditions was performed for primer, template DNA and $MgCl_2$ concentrations. An optimal primer concentration of 300 nm and a final $MgCl_2$ concentration of 3 mM were finally chosen for the assay under the following cycle conditions: one cycle of 50 °C for 2 min and 95 °C for 2 min for initial denaturation, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each denaturation and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by increasing the temperature at a rate of 1 °C every 30 s from 60 to 95 °C. Total microbial rumen DNA was diluted to 1 : 10 prior to use in real-time PCR assays to reduce inhibition.

Real-time PCR calibrations

Standard curves for the absolute quantification of *F. succinogenes* and *R. flavefaciens* were obtained using DNA from *F. succinogenes* S85 and *R. flavefaciens* Y1 grown overnight in culture at 39 °C. Cultures were centrifuged and the cells were resuspended in clarified rumen fluid. The numbers of bacterial cells were determined using a Helber counting chamber (Weber Scientific Instruments, West Sussex, UK) at × 400 magnification. DNA was then extracted from a known number of cells and used in a 6 log dilution series.

Standard curves for anaerobic fungi were obtained using DNA from two monocentric fungi, *Neocallimastix* sp. CX and *Piromyces* sp. TGB1, and a polycentric isolate, *Orpinomyces* sp. F11, from 30-h cultures. The dry weight biomass was calculated for each fungal isolate and resuspended in clarified rumen fluid prior to DNA extraction as described above. DNA was then diluted to generate six concentrations over a 3 log range. Standard curves were also generated for each fungus separately.

Statistics

Statistical analysis of the data was performed by ANOVA, with differences determined by the method of least significant differences at the 5% level ($P < 0.05$). All statistical analyses were run with Statistica 6 (StatSoft Inc., Tulsa, OK).

Results

Anaerobic fungal growth curves

The polycentric isolate *Orpinomyces* sp. F11 was observed to generate significantly greater biomass across all time points after 12 h of growth compared with the monocentric isolates. The final dry weight biomass for *Orpinomyces* sp. F11 was 12.9 mg at 36 h for a 10-mL culture, whereas the average of the monocentric fungal isolates was 2.4 mg (Fig. 1).

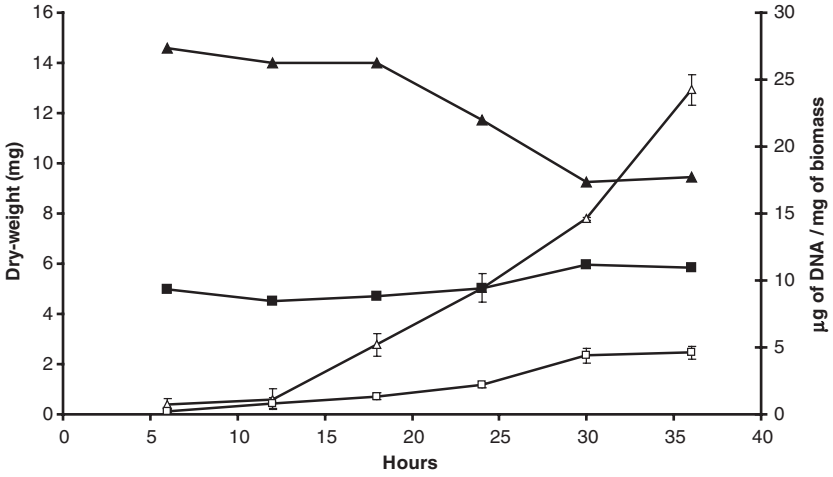

Fig. 1. Growth curves showing biomass gain for the polycentric isolate *Orpinomyces* sp. F11 (△) and the average for the monocentric isolates *Neocallimastix patriciarum* CX and *Piromyces* sp. TGB1 (□). The DNA content per milligram of biomass is represented as filled symbols for the respective isolates.

Crude protein estimations per milligram of biomass, performed at each time point, revealed that both the monocentric and polycentric isolates possessed similar crude protein levels of 40.5% and 41%, respectively. Across the growth period, the crude protein coefficient of variance was 0.8% for the polycentric fungal isolate and 3.8% for the monocentric fungi.

DNA was extracted from a known quantity of biomass at each time point to ascertain the ratio of DNA to fungal biomass. The monocentric fungi exhibited similar values at all time points, with an average of $9.7 \pm 0.45\,\mu g\,mg^{-1}$ (mean ± SEM) and with a variance of 1.2%. The polycentric fungus, however, showed higher values within the first 18 h, with a mean of $26.6 \pm 0.37\,\mu g\,mg^{-1}$ (mean ± SEM), whereas, in the later stages of growth, the DNA content was, on average, $19.02 \pm 1.49\,\mu g\,mg^{-1}$ (mean ± SEM; Fig. 1). The coefficient of variance across the entire growth period was 20.2%, compared with 0.4% for the first 18 h and 6.7% for the remaining 18 h of growth.

Real-time PCR performed on 10 ng of fungal genomic DNA at each time point produced a consistent cycle threshold (C_t) value of 13.82 ± 0.09 (mean ± SEM) for the polycentric isolate and 14.74 ± 0.27 (mean ± SEM) for the monocentric isolates.

PCR primer design and validation

The general anaerobic fungal forward primer was designed against conserved regions of the 3′ end of the 18S ribosomal gene, whereas the reverse primer was designed within the 5′ region of the ITS1 using multiple alignments of this region (Brookman *et al*., 2000). The amplicon size for the anaerobic fungal primers was *c*. 120 bp. Amplicons were only observed for DNA extracted from the nine anaerobic fungal strains and from total rumen microbial DNA. PCR amplification of rumen microbial DNA using the anaerobic fungal primers produced fungal-specific amplicons of the expected size. Cloning and sequencing of these amplicons revealed that all products examined aligned with anaerobic rumen fungal sequences (data not shown). No product was observed for samples extracted from nine other closely related aerobic chytrid fungi (Table 1).

The primer sets designed to detect the two fibrolytic anaerobic rumen bacterial species *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were designed to target their respective 16S ribosomal gene. Results from probe match analysis at RDP II and within ARB both showed the primers to be specific for their respective targets. Because of the need to limit the amplicon size, the forward primer in the design of the *F. succinogenes* primers was found to be incompletely target specific; specificity for the target was achieved through the reverse primer sequence site. The reverse primer sequence was found to be highly specific for *F. succinogenes* when tested against the NCBI, RDP II and ARB databases. When tested against DNA from pure bacterial cultures, the *F. succinogenes* primer set was observed to be target specific (Table 1). In addition, the *F. succinogenes* primer set was shown to be capable of amplifying strains from all three *F. succinogenes* subgroups through the amplification of subgroup representative strains BL2, S85, HM2 and REH9-1 (Lin *et al*., 1994). PCR amplification of cattle rumen microbial DNA using the specific bacterial primer sets produced amplicons of the expected size. Cloning and limited sequencing of these amplicons revealed that all products examined aligned with *F. succinogenes* and *R. flavefaciens* sequences with respect to the primers used.

The general 16S ribosomal bacterial primer set was designed as a modified version of an existing 16S universal primer (Lane, 1991); the second primer was designed at a distance of up to 200 bp from the universal primer. When considering the design of the second primer, multiple alignments of representative 16S rumen bacteria and those

obtained from RDP II (Cole *et al.*, 2003) were analysed in the ARB software environment for sequence database handling and data analysis (Ludwig *et al.*, 2004). Final primer sequences were tested against the RDP II and ARB multiple alignment using the probe match analysis functions in the respective databases. When limiting searches to near-complete sequence lengths and allowing for up to two mismatches, *c.* 95% of all sequences were detected. The amplicon size for the primer set was *c.* 130 bp.

Validation of real-time PCR assay

Dissociation curve analysis to ascertain the T_m of amplicons is an essential element for determining the specificity of a SYBR Green assay. Dissociation analysis of amplicons was performed for all primer sets using DNA from pure cultures and total rumen microbial DNA. The primers for *R. flavefaciens*, when used with DNA from strain Y1, produced a dissociation curve peak at *c.* 82 °C; an identical peak was observed when using rumen microbial DNA. A dissociation curve peak of 86 °C was observed for the *F. succinogenes* primer set when either the pure DNA from strain S85 or rumen microbial DNA was used. When using the general bacterial primer set with total rumen microbial DNA, a broad dissociation curve was observed at 82–89 °C. The general anaerobic fungal primers were designed to target a highly conserved region, thus producing amplicons of similar sequence composition across target species, resulting in a more defined dissociation peak. The general rumen fungal primers, when used with either pure anaerobic fungal DNA or rumen microbial DNA, produced dissociation curves at 76 °C.

Standard curve generation

Standard curves for the two bacterial targets were generated by plotting the observed C_t values against the logarithm of the cell number per millilitre. Standard curves from the various pure cultures of anaerobic fungi produced similar slopes, but resulted in different *y*-intercepts, with the polycentric isolate having a lower intercept. The difference in these intercepts divided by the slope (0.34) estimates the target gene to be double in the polycentric isolate than in the monocentric isolate. In addition, when 10 ng of DNA from pure cultures taken throughout the growth study was used in the real-time PCR assay, the average difference in C_t values between the polycentric and monocentric isolates across all time points was 0.91 ± 0.18 (mean ± SEM). This also equates to a difference of approximately twofold between the isolates. Therefore, standard curves of anaerobic fungi were generated by plotting the observed C_t values against the logarithm of the number of micrograms of a mixed fungal DNA dilution. Standard curve slopes are presented in Table 3, revealing that all primer sets, regardless of DNA template, from pure cultures, mixed cultures or rumen samples produced PCR efficiencies approaching 100%. To convert DNA values to fungal biomass, an average DNA content of 13 μg for every milligram of total rumen fungal biomass was used.

Table 3. Standard curve line fit and slope values calculated using real-time PCR

	R^2	Slope
Fibrobacter succinogenes	0.999	−3.386
	0.999	−3.467
Ruminococcus flavefaciens	0.997	−3.340
	0.995	−3.370
General anaerobic fungi	0.996	−3.384
	0.993	−3.354
	0.998	−3.233
	0.997	−3.285
	0.991	−3.292
General bacteria	0.999	−3.327

Values for each bacterial primer set are for total microbial rumen DNA template (top row) and the respective pure culture (bottom row). Fungal DNA template was as follows for top row: total microbial rumen DNA template, *Orpinomyces* sp. F11, *Piromyces* sp. TGB1, *Neocallimastix* sp. CX and an equal mix of all three fungal templates.

Real-time PCR monitoring of microbial populations within the rumen

Using absolute quantification, *R. flavefaciens* was observed to be present at a consistent level of $(1.15 \times 10^8) \pm (0.29 \times 10^8)$ cells mL^{-1} (mean ± SEM), and was not observed to be significantly different at any sampling time (Fig. 2). Monitoring of the *F. succinogenes* population over the 24-h sampling period showed significant changes from each sampling point, with a general increase in detection to $(7.91 \times 10^9) \pm (2.04 \times 10^9)$ cells mL^{-1} (mean ± SEM) at 12 h after feeding (Fig. 2). *Fibrobacter succinogenes* was observed to increase significantly by 3.13-fold ($P < 0.01$) from 4 to 8 h, with a further increase of 1.16-fold for the 12-h sampling time. A similar diurnal pattern for the general anaerobic fungal population was revealed, with a significant increase from 4 to 8 h of 2.15-fold ($P < 0.01$), and then a further increase of 1.10-fold to the 12-h sampling time (Fig. 2). The fungi reached a maximum of 22.03 ± 2.74 μg of biomass mL^{-1} (mean ± SEM) at 12 h after feeding. This equates to a crude protein value of 8.98 ± 1.12 μg mL^{-1} (mean ± SEM).

A second method of monitoring the population changes was also investigated: through the use of a relative quantification assay. In this instance, the total 16S rRNA gene amplified by the total bacterial primer set was used as a 'housekeeping gene' for normalization of the data. As the two quantification methods result in different values, the

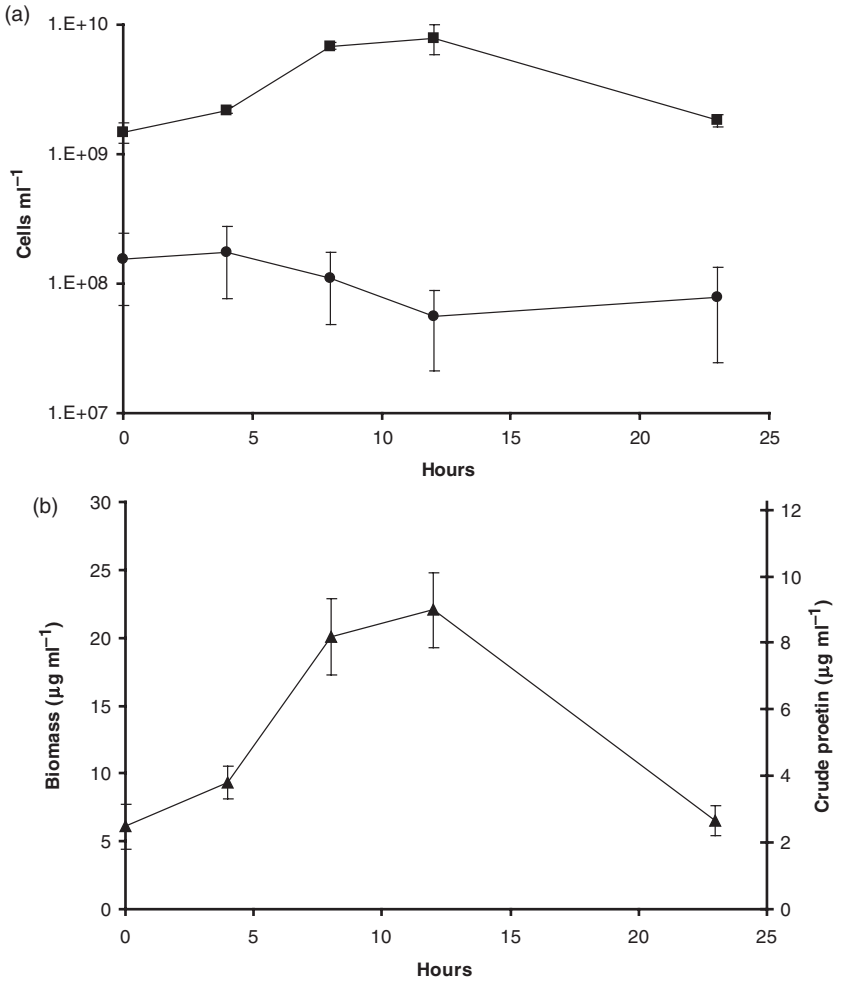

Fig. 2. Real-time PCR data representing specific targets: (a) *Fibrobacter succinogenes* (■) and *Ruminococcus flavefaciens* (○); (b) total anaerobic fungi (▲). Points represent the mean of four animal samples; error bars are standard errors of the mean. Time represents hours at which samples were collected after feeding.

Table 4. Comparison of population changes between time points using absolute or relative quantification calculations

	0–4 h	4–8 h	8–12 h	12–24 h
F. succinogenes	1.8 ± 0.52	4.26 ± 0.75	1.19 ± 0.15	0.43 ± 0.03
	1.69 ± 0.27	2.83 ± 0.45	1.06 ± 0.20	0.31 ± 0.06
R. flavefaciens	1.13 ± 0.21	0.83 ± 0.09	0.67 ± 0.07	2.20 ± 0.42*
	1.14 ± 0.05	0.64 ± 0.09	0.49 ± 0.03	1.34 ± 0.13*
Fungi	2.53 ± 1.34	2.96 ± 0.36	1.29 ± 0.07	0.50 ± 0.08
	2.25 ± 0.94	2.18 ± 0.30	1.17 ± 0.23	0.31 ± 0.06

*Significant difference using Tukey HSD calculations.
Values show the mean fold difference ± SEM in the target detected between the time points indicated. Top row values were calculated using a relative quantification assay with total bacterial values for normalization. Bottom row values were calculated as absolute values from standard curve generation.

differences between consecutive time points (calculated by each method) for each population were used to investigate any significant difference between the methods. There was no observed significant difference between the methods for any of the populations (Table 4). Only when using Tukey honestly significant difference (HSD) calculations to overcome a nonsignificant *F*-test was *R. flavefaciens* observed to be significantly different ($P < 0.05$) between the methods for the 12–24-h period. Further analysis by comparing the difference between the two fibrolytic bacterial populations at each time point did not show any significant difference between the methods (Fig. 3).

Discussion

Anaerobic rumen filamentous fungi form extensive interlocking rhizoidal systems. Accurate enumeration and identification within these masses by direct count using a microscope is not practical, especially when considering that environmental samples contain both polycentric and monocentric species. Counting zoospores or thallus structures alone is not entirely accurate, particularly when polycentric fungi can form colonies from fragments of rhizomycelium in addition to zoospores (Dehority & Orpin, 1997). An

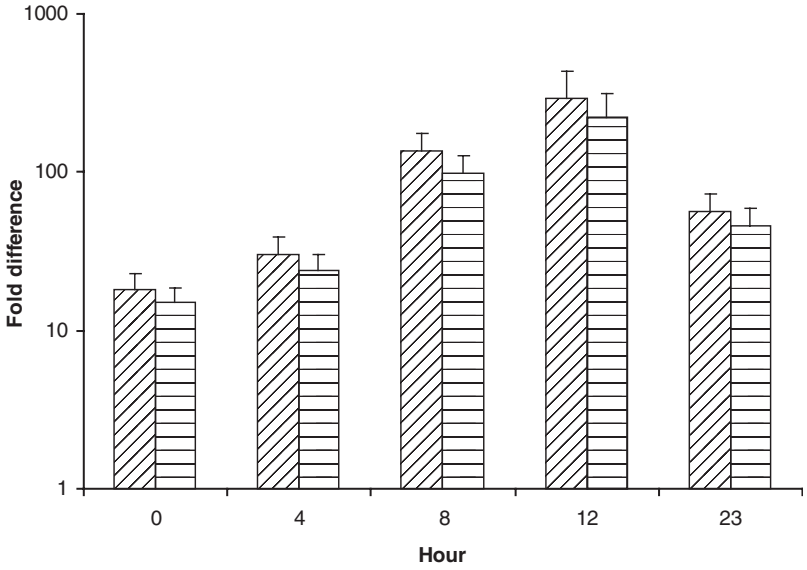

Fig. 3. Comparison of fold difference between *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* at the indicated time points after feeding using relative (▨) or absolute (▤) quantification calculations.

MPN method was developed (Theodorou *et al.*, 1990; Obispo & Dehority, 1992) that consistently yielded higher fungal counts when compared with direct microscopy counts (Dehority & Orpin, 1997). A drawback of this method is that the fungi grow as a nonhomogeneous culture in liquid media and therefore cannot be accurately serially diluted. In particular, some polycentric fungal isolates form a thick pellicle structure when grown *in vitro* which is difficult to disrupt (personal observations).

To overcome this, data are presented with respect to fungal biomass or crude protein per millilitre. In order for this to be valid, an understanding of DNA and crude protein content from representatives of both polycentric and monocentric anaerobic fungi during various growth stages is required. Although the monocentric pure cultures showed no significant difference in the proportion of DNA to fungal biomass, the polycentric isolate clearly possessed a higher DNA content in the earlier stages of growth than in the later stages. This coincides with the growth characteristics of *Orpinomyces* spp. in that, during the initial stages of growth, an extensive polynucleated rhizomycelium is evident (Breton *et al.*, 1989). The decline in the DNA to biomass ratio suggests that, in the later stages of growth, enlargement of the rhizomycelium occurs without any major nuclear division.

The cattle rumen is known to possess both polycentric and monocentric species, and therefore a mean value of 13 $\mu g\,mL^{-1}$ of biomass was employed for converting DNA content to biomass and then to fungal nitrogen or crude protein values. This value was chosen as the average DNA content for the various fungal species during active growth, so as to allow for the observed differences across the fungal growth stages and between the two major forms. In systems in which only monocentric species are present, a lower value of *c.* 10 $\mu g\,mL^{-1}$ of biomass should be employed, whereas, in environments predominantly inhabited by polycentric species, a higher value approaching 20 $\mu g\,mL^{-1}$ of biomass is required. There was no significant difference in nitrogen values between polycentric and monocentric fungi, and they were similar at various stages of fungal growth; an average value of 6.5% was used. This value is similar to that mentioned previously for anaerobic fungi of 5.9% nitrogen (Faichney *et al.*, 1997).

The general fungal primer set was found to amplify all anaerobic fungal DNA across the three genera tested, but did not amplify any of the aerobic fungal isolates. None of the four representatives from the *Spizellomycetales* order, which is the most closely related order to the anaerobic fungi (Bowman *et al.*, 1992), or of the representatives of the other related orders that were tested, produced an amplicon when using the anaerobic fungal-specific primer set. The aerobic chytrid fungi are commonly found and cultured from soils (Sparrow, 1960), and are particularly prevalent in soils subjected to agriculture and horticultural practices (Weber & Webster, 2000). It is most likely that these fungi would be ingested by grazing animals and could therefore be present within samples from grazing animals. Soil and grass samples collected from a nongrazing site did not produce an amplification product. A nongrazing site was chosen in order to reduce potential interference from any aero-tolerant survival stage of the anaerobic fungal strains (Davies *et al.*, 1993).

Earlier studies estimating fungal biomass in the rumen using chitinase postulated the fungal biomass to be around 8% of the biomass (Orpin & Joblin, 1988). The authors pointed out that this assay is susceptible to a large amount of

error, and there is likely to be interference from feedstuffs and aerobic fungal contamination. Fungal abundance calculated by various counting methods is usually within the range 10^3–10^7 mL^{-1} as zoospores or TFUs (Theodorou, 1990; Orpin & Joblin, 1997). In sheep fed an orchard grass diet, Faichney *et al.* (1997) used an rRNA-based probing method to calculate the fungal contribution to be between 1.1% and 3.5% of microbial nitrogen. Studies employing rRNA quantification are susceptible to error because of changes in the rRNA content throughout the cell growth stages (Raskin *et al.*, 1997). The rDNA content, however, remains more constant, as was observed here, with no significant changes in target values over the growth cycle of the various fungal isolates. Similar observations have been reported, supporting the view that rDNA is a more robust target (Blomberg *et al.*, 1997; Sylvester *et al.*, 2004). In this study, the estimation of rumen microbial nitrogen based on total purine analysis, organic dry matter digestibility and rumen turnover rate was 14.9 g nitrogen kg^{-1} (P. Kennedy, CSIRO, Rockhampton, Qld, Australia, unpublished data). This then equates the rumen fungal contribution to total microbial nitrogen at 0.5% in these animals 12 h after feeding. With these animals on a low-quality diet, which was also deficient in sulphur, it is likely that the fungal population would be lower than on better quality diets such as orchard grass. Rumen fungal numbers have previously been observed to increase in response to sulphur supplementation of sheep fed a deficient diet of spear grass (*Heteropogon contortus*) (Morrison *et al.*, 1990).

The primer sets selected to monitor the fibrolytic bacterial species *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in this trial were specific for the respective targets and produced no cross-reactivity with the other species tested. Dissociation curve profiles for both *F. succinogenes* and *R. flavefaciens* products were tight, suggesting a high level of specificity. These primers are designed within variable regions in the 16S ribosomal gene and, therefore, any nonspecific amplification from other rumen microbial populations would result in erroneous dissociation curves. In order to overcome any potential inhibitory compounds from rumen fluid, which have been observed previously to affect standard curve generation (Ouwerkerk *et al.*, 2002), standard curves were generated from harvested cells that were first resuspended in clarified rumen fluid prior to DNA extraction. The *F. succinogenes* primer set described here produces a smaller amplicon (121 bp) than that previously described (445 bp) (Tajima *et al.*, 2001). Similarly, the primer set described here for *R. flavefaciens* was reduced to an amplicon size of 132 bp to maintain amplification efficiency. The amplicon size should be kept to a minimum, thus overcoming amplification problems associated with larger fragments. The *F. succinogenes* 16S rRNA gene was observed by Tajima *et al.* (2001) to be the least efficiently amplified of the rumen bacteria when the entire 16S gene was amplified.

Previous studies monitoring fibrolytic bacterial populations within the rumen through various molecular-based methods have indicated that *F. succinogenes* is present as the predominant fibrolytic species at *c.* 2% of the population (range, 0.1–6%) with respect to the total microbial rRNA signal (Stahl *et al.*, 1988; Briesacher *et al.*, 1992; Lin *et al.*, 1994; Krause *et al.*, 1999; Tajima *et al.*, 2001; Koike *et al.*, 2003). Similarly, the data gathered here suggest that *F. succinogenes* is present at high numbers within the rumen and significantly increases to a maximum at 12 h after feeding. The use of these primers to track these populations and their responses to diet changes has not been performed here, and so little comparison can be undertaken in the context of this paper, other than to recognize that *F. succinogenes* was present at 2 $\log_{10}$ numbers greater than *R. flavefaciens*. It was also observed that, although *R. flavefaciens* was not significantly different at any time point, it was detected at its lowest value when *F. succinogenes* was observed to be present at its highest level.

In addition to absolute quantification, a relative quantification assay was performed on the same samples, in which the samples were normalized against a total 16S ribosomal target so as to account for any difference in sample processing. This is akin to relative quantification of target genes in gene expression studies. This means that only the difference between treatments can be observed or, in this case, species shifts between time intervals. When comparing the calculated fold differences detected for the target species for the two methods, the relative quantification was observed to generally produce higher values, but was not observed to be significantly different from the absolute quantification methods. The relative quantification assay may be of use in applications in which the tracking of multiple targets within many samples and the running of multiple standard curves with each assay become prohibitive. This type of assay can only show population changes with respect to samples and cannot provide absolute cell numbers.

The development of the anaerobic fungal real-time PCR assay allows researchers, for the first time, to more rapidly and accurately monitor total anaerobic fungal populations within the rumen. It is envisaged that, in future studies, it will be possible to monitor specific subpopulations using species-specific primers from signature sequences recently elucidated (Tuckwell *et al.*, 2005).

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