

# Dynamics of initial colonization of nonconserved perennial ryegrass by anaerobic fungi in the bovine rumen

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## Keywords

*Neocallimastigales*; rumen; colonization; ITS1; 5.8S rRNA gene.

## Abstract

Anaerobic fungi (*Neocallimastigales*) are active degraders of fibrous plant material in the rumen. However, only limited information is available relating to how quickly they colonize ingested feed particles. The aim of this study was to determine the dynamics of initial colonization of forage by anaerobic fungi in the rumen and the impact of different postsampling wash procedures used to remove loosely associated microorganisms. *Neocallimastigales*-specific molecular techniques were optimized to ensure maximal coverage before application to assess the population size (quantitative PCR) and composition (automated ribosomal intergenic spacer analysis) of the colonizing anaerobic fungi. Colonization of perennial ryegrass (PRG) was evident within 5 min, with no consistent effect of time or wash procedure on fungal population composition. Wash procedure had no effect on population size unlike time, which had a significant effect. Colonizing fungal population size continued to increase over the incubation period after an initial lag of *c.* 4 min. This dynamic differs from that reported previously for rumen bacteria, where substantial colonization of PRG occurred within 5 min. The observed delay in colonization of plant material by anaerobic fungi is suggested to be primarily mediated by the time taken for fungal zoospores to locate, attach and encyst on plant material.

## Introduction

Anaerobic fungi (order *Neocallimastigales*) are active degraders of fibrous plant material in the rumen, and although knowledge is limited, some researchers consider that they are primary colonizers of plant biomass in this environment. Joblin *et al.* (1989) found that solubilization of fibrous plant material by anaerobic fungi was more extensive than by cellulolytic rumen bacteria. This is thought to be due to their broad range of potent polysaccharide-degrading enzymes (Orpin & Joblin, 1997), as well as their ability to penetrate complex structural barriers (Ho *et al.*, 1988; Joblin, 1989), physically opening up internal plant tissues to enzymatic attack. Anaerobic fungi therefore are likely to play an important role in the ruminal degradation of complex recalcitrant lignocellulosic material.

Colonization of plant biomass by motile zoospores (5–10 µm in diameter) is a key step in the degradation

process and is representative of a critical step in the life cycle of the anaerobic fungi, preferentially colonizing sites of damage on plant material. Several studies have shown that ingested forage is colonized within 15–20 min of its ruminal incubation (Orpin, 1977a; Bauchop, 1980; Ho *et al.*, 1988). Once attached, zoospores encyst on plant material before germinating and developing a vegetative rhizoidal system, which ultimately supports the developing reproductive sporangium (Lowe *et al.*, 1987).

Rumen bacteria also preferentially colonize sites of damage on plant material (Latham *et al.*, 1978), with diverse bacterial populations colonizing within 5 min of ruminal incubation (Edwards *et al.*, 2007). Interactions between anaerobic fungi and the other microbial taxa resident within the rumen have been extensively studied *in vitro*, particularly with hydrogen-utilizing microorganisms (Wood *et al.*, 1986; Marvin-Sikkema *et al.*, 1990; Joblin & Williams, 1991). Ruminal cellulolytic bacteria have been shown to colonize

plant material rapidly (Koike *et al.*, 2003), and have a variable effect on anaerobic fungal growth and/or activity (Williams *et al.*, 1994). Cocultivation of anaerobic fungi with *Fibrobacter succinogenes* enhanced the overall digestion of plant material, whereas cocultivation with *Ruminococcus* spp. had a negative effect (Williams *et al.*, 1994). However, it is interesting to note that *in vitro* studies with different bacterial strains of the same species (*Selenomonas ruminantium*) have generated contrasting results (Orpin & Joblin, 1997).

Recent improvements in molecular techniques have enabled the analysis of the rumen bacterial populations initially colonizing newly ingested fresh forage in the rumen, in terms of both population size and composition (Edwards *et al.*, 2007). The population size and composition of anaerobic fungal populations can also be analysed using PCR-based approaches targeting the *rrn* operon. Quantitation of *Neocallimastigales* populations using a quantitative PCR (QPCR) approach has been reported previously, using primers targeting the end of the 18S rRNA gene and beginning of the internal transcribed spacer 1 (ITS1) region (Denman & McSweeney, 2006). Anaerobic fungal population composition has been assessed previously using the ITS1 size polymorphism, which although it only has an approximate relationship with genus, is sufficiently robust to monitor changes in *Neocallimastigales* populations (Brookman & Nicholson, 2005). Unlike other eukaryotic populations, anaerobic fungal genera cannot be differentiated effectively based on the 18S rRNA gene (Brookman *et al.*, 2000).

Improved fundamental knowledge about the progression of colonization, the first key step in the process of microbial degradation of plant material, will contribute to identification of limitations that impact on rumen function. This study focused on characterizing the initial colonization of

fresh forage by anaerobic fungi directly within the rumen using an *in sacco* approach, with the use of molecular techniques to enable fungal colonization of bag residues to be specifically studied in the presence of other rumen microbiota. As sample-washing methods, used to remove loosely associated microorganisms, have been shown to potentially influence the apparent colonization by bacteria (Edwards *et al.*, 2007), the effect of the washing method on the analysis of colonization by anaerobic fungi was also assessed. Further development of existing techniques (Brookman & Nicholson, 2005; Denman & McSweeney, 2006) was also conducted as part of this study to ensure maximal coverage of the anaerobic fungi with the molecular techniques used.

## Materials and methods

### Anaerobic fungal cultures and rumen content

Axenic cultures of anaerobic fungi were used for validation of methods and as QPCR standards. The following cultures were obtained from the Institute of Grassland and Environmental Research culture collection (Aberystwyth, UK): SR2, W7, PC2-2, C2-2H and C1. *Neocallimastix frontalis* (R<sub>E</sub>1) was kindly provided by Dr R. John Wallace from stock cultures held within the Rowett Research Institute (Aberdeen, UK). *Caecomyces* sp. Isol1 was isolated from the rumen content of a nonlactating rumen-fistulated Holstein-Friesian cow fed a barley straw diet; rumen content was also directly frozen for later DNA extraction. Other cultures (GE01, 09, 21 and 42) were isolated by Mr Gary Easton and Dr David Davies using standard isolation procedures (Theodorou *et al.*, 2005). Further details of the fungal cultures are provided in Table 1.

**Table 1.** Cultures used in this study

Strain (isolate code*)	Genus	Morphology			Origin (host, country)
		Thallus	Rhizoid	Zoospore	
W7 (NCS1)	<i>Neocallimastix</i>	MonoC	Filamentous	PolyF	Goat, China
GE21	<i>Neocallimastix/Piromyces</i>	MonoC	Filamentous	–	Sheep, UK
R <sub>E</sub> 1	<i>Neocallimastix</i>	MonoC	Filamentous	PolyF	Sheep, UK
PC2-2 (NMW5)	<i>Neocallimastix</i>	MonoC	Filamentous	PolyF	Buffalo, Malaysia
C2-2H	<i>Neocallimastix</i>	MonoC	Filamentous	PolyF	Cow, Malaysia
C1 (PCS1)	<i>Piromyces</i>	MonoC	Filamentous	MonoF	Sheep, China
GE01	<i>Orpinomyces</i>	PolyC	Filamentous	PolyF	Sheep, UK
SR2 (OUS1)	<i>Orpinomyces</i>	PolyC	Filamentous	PolyF	Sheep, UK
GE09	<i>Anaeromyces</i>	PolyC	Filamentous	–	Buffalo, UK
GE42	<i>Caecomyces</i>	PolyC	Bulbous	–	Sheep, UK
Isol1	<i>Caecomyces</i>	MonoC	Bulbous	–	Cow, UK

\*Isolate code as described previously by Brookman *et al.* (2000).

–, not determined; MonoC, Monocentric; PolyC, polycentric; PolyF, polyflagellated; MonoF, monoflagellated.

## Rumen *in sacco* experiment with perennial ryegrass

All animal experiments were conducted with the authority of licenses held under the UK Animal Scientific Procedures Act, 1986. Two nonlactating rumen-fistulated Holstein–Friesian cows (A and B) grazing a perennial ryegrass sward were used. Six-week regrowth material of *Lolium perenne* cv. Aberdart was collected in July from an ungrazed field plot (maintained under natural conditions) on the morning of the experiment using mechanical shears held 5 cm above the ground level. The plant material was then chopped (c. 2–5 cm length) with an industrial bowl chopper (Lynhacker GH33, George Hansen, Copenhagen, Denmark) to mimic mastication, as described previously (Kim *et al.*, 2005). Polyester bags (22 × 9 cm with 40-µm mesh pore size) were loosely filled with 10 g (fresh weight) of the processed plant material. Forty bags were prepared in total, of which 16 were incubated in the rumen of each cow (i.e. 32 bags). Bags were attached to 50 cm semiflexible stalks so as to retain them within the liquid phase of the ruminal content. At each time point (5, 10, 15 and 30 min), four bags were removed from each cow: two processed by machine washing and two by hand washing. For the 0-min time point, filled polyester bags were processed directly for washing without entering the rumen. Machine washing (Hotpoint Electronic 1000 Plus 9534) of duplicate bags involved two rinse cycles (2 × 13.5 L) and a final spin cycle. For hand washing, bag contents were removed and placed in a sieve (1-mm mesh size), rinsed twice with 2 L of cold water and then hand squeezed. Washed samples were then snap frozen in liquid N<sub>2</sub>, ground by a mortar and pestle and stored at –80 °C for later DNA extraction.

## Extraction of DNA

DNA from ground plant material (200 mg fresh weight), freeze-dried and ground rumen content (20 mg dry weight) and axenic cultures was extracted using the BIO101 FastDNA Spin Kit for soil (QBiogene, Cambridge, UK). The manufacturer's guidelines were followed, with the exception that the samples were processed for 3 × 30 s at speed 6.0 in the FastPrep instrument (QBiogene), with incubation for 30 s on ice between pulses of bead beating. The integrity of the DNA was verified by agarose gel electrophoresis and DNA was quantified using bisbenzimidazole (Sambrook & Russell, 2001).

## *Neocallimastigales*-specific automated ribosomal intergenic spacer analysis (ARISA)

Amplification of the *Neocallimastigales* ITS1 region was carried out with the forward primer Neo 18S For (5'-6FAM AAT CCT TCG GAT TGG CT-3') and Neo 5.8S Rev (5'-

CGA GAA CCA AGA GAT CCA-3'). All PCR amplifications were performed using a 2720 thermal cycler (Applied Biosystems, Warrington, UK) in 10 µL volumes containing 1 × PCR buffer (40 mM Tricine-KOH pH 8.0, 16 mM KCl, 3.5 mM MgCl<sub>2</sub> and 3.75 µg mL<sup>-1</sup> bovine serum albumin), 800 µM dNTP, 500 nM of each primer, 0.2 µL of 50 × Titanium Taq DNA polymerase (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) and c. 20 ng of DNA. Amplification conditions for the primer pair were as follows: an initial denaturation of 95 °C for 5 min followed by 10 cycles of 95 °C 30 s, 68 °C (–1 °C each cycle) 30 s and 72 °C 30 s; 25 cycles of 95 °C 30 s, 58 °C 30 s, 72 °C 30 s; and a final extension of 72 °C for 6 min. After PCR, successful amplification was verified by checking for products (c. 350–450 bp) by agarose gel electrophoresis.

For each sample, triplicate PCR reactions were performed and then combined. Each combined PCR product was then diluted with molecular-grade water, and 1.0 µL was mixed with 0.25 µL of GeneScan 500 LIZ size standard (Applied Biosystems) and 9.75 µL of Hi-Di<sup>TM</sup> Formamide (Applied Biosystems). The mixture was then denatured for 5 min at 95 °C and cooled on ice before being run on an ABI 3130xl Genetic Analyser (Applied Biosystems) with a 36 cm array, Performance Optimized Polymer-7 and dye set G5. Spectral data were then exported from GENEMAPPER (Applied Biosystems) as a table of peaks and analysed with the FINGERPRINT TYPE and CLUSTER ANALYSIS modules of the FINGERPRINTING software package (Bio-Rad UK Ltd, Hemel Hempstead, UK). Cluster analysis was performed using both the Pearson (curve based, i.e. peak intensity) and the Dice (band based, i.e. peak presence/absence) algorithms with a position tolerance of 0.4% and an optimization parameter of 0.5%. Peaks < 3% of the maximum peak area were included in the analysis as 'uncertain' due to sample background (i.e. standard pull-ups and PCR-based artefacts), so that their presence/absence would not penalize cluster analysis.

## *Neocallimastigales*-specific QPCR

QPCR was performed with a 7500 real-time PCR system (Applied Biosystems) using the primer pair Neo QPCR For (5'-TTG ACA ATG GAT CTC TTG GTT CTC-3') and Neo QPCR Rev (5'-GTG CAA TAT GCG TTC GAA GAT T-3'), targeting a conserved region (110 bp) of the 5.8S rRNA gene, along with a Taqman probe (Neo: 5'-6-FAM CAA AAT GCG ATA AGT ART GTG AAT TGC AGA ATA CG TAMRA-3'). The reaction mixture (25 µL) contained 1 × Taqman Universal PCR Master Mix (Applied Biosystems), 750 nM of each primer, 200 nM of the probe and a 2 µL volume of template DNA (c. 20 ng). The thermal cycling programme was 40 cycles of 95 °C for 15 s and 60 °C for 1 min with an initial cycle of 95 °C for 2 min after incubation at 50 °C for 2 min. During each cycle, accumulation of PCR products was detected by monitoring the fluorescence signal from the

probe. An anaerobic fungal DNA standard was prepared with equal amounts of genomic DNA from three different axenic cultures of anaerobic fungi that were diverse in their morphology [*N. frontalis* (R<sub>E1</sub>), *Orpinomyces* sp. SR2 and *Caecomycetes* sp. Isol1] in a carrier DNA solution (12.5 µg mL<sup>-1</sup> herring sperm DNA). The carrier DNA solution was used as a nontemplate control. All raw data were analysed using 7500 System SDS software (Applied Biosystems). The QPCR data obtained were normalized based on the chlorophyll content of the samples as described previously (Edwards *et al.*, 2007), as the moisture content of the samples differed between wash treatments. Assay PCR efficiency was calculated as follows: efficiency =  $(10^{-1/\text{slope}} - 1) \times 100$ .

#### Statistical analysis of *in sacco* experiment QPCR data

Mean values ( $n=4$ ) were calculated for each washing method at each time point above 0 min. The effects of washing method and incubation time were examined assuming an asymptotic relationship between the quantity of *Neocallimastigales* DNA and incubation time course. An exponential model was fitted to the data for time points > 0 min with the aid of GENSTAT 10th edition (Payne, 2006).

#### Cloning and sequence analysis of *Neocallimastigales* PCR amplicons

The specificity of the ARISA primer pair for ruminal *Neocallimastigales* was confirmed by cloning and sequencing a PCR product generated from rumen content collected from a nonlactating rumen-fistulated Holstein–Friesian cow fed a barley straw diet. The PCR product was cleaned using a QIAquick PCR purification kit (Qiagen, West Sussex, UK) following the manufacturer's guidelines and cloned into *Escherichia coli* using the pGEM-T Easy Vector System (Promega, Southampton, UK). Twenty-two transformed colonies, containing an insert within the predicted size range (c. 350–450 bp), were sequenced. Sequence determination was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI3130xl Genetic Analyser.

Cloning and sequence analysis of the *Anaeromyces* sp. GE09 amplicon (partial 18S rRNA gene, full ITS1, full 5.8S rRNA gene and partial ITS2) generated using primers GM1 (5'-TGTACA CAC CGC CCG TC-3', Brookman *et al.*, 2000) and MN106 (5'-CGT TGT AAA ACA CTC AWA ACC-3'; Nicholson, 2003) was performed in a similar manner, except that five clones were sequenced. Sequence data generated in this study have been submitted to the GenBank database under accession numbers EU414755–EU414770.

## Results

### Development of *Neocallimastigales*-specific ARISA and QPCR methods

A previously developed primer pair for *Neocallimastigales* ITS1-based size polymorphism analysis (Brookman & Nicholson, 2005) was found to have limited coverage of the order. Partial sequence analysis of the *Anaeromyces* sp. GE09 *rrn* operon (EU414755–EU414759) demonstrated that the forward primer MN100 (5'-TCC TAC CCT TTG TGA ATT TG-3') had four mismatches with this axenic culture. Alignment of the primer pair with available *Neocallimastigales* sequence data [deposited in the National Centre for Biotechnological Information (NCBI) database] also demonstrated that the forward primer MN100 had one or two mismatches with c. 10% of the sequences currently deposited. These findings indicated that the area of the ITS1 region targeted by MN100 was not conserved.

A new ARISA primer pair was designed that targeted the end of the 18S rRNA gene (Neo 18S For) and the start of the 5.8S rRNA gene (Neo 5.8S Rev). As the reverse primer of the available *Neocallimastigales* specific QPCR method was also based on the same ITS1 region targeted by the MN100 primer (Denman & McSweeney, 2006), a probe-based QPCR assay targeting the 5.8S rRNA gene was developed. *In silico* analysis using available *Neocallimastigales* sequence data (NCBI) and BLAST analysis demonstrated that the primer pairs and probe gave good specificity and coverage for all the six currently recognized genera, as well as deposited environmental sequences. The *Neocallimastigales* ARISA amplicon was predicted to give fragments ranging in size from 357 to 441 bp (data not shown).

### Validation of *Neocallimastigales*-specific ARISA and QPCR methods

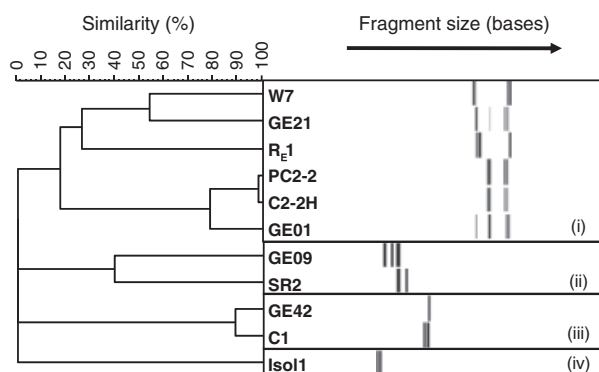
Application of the ARISA primers to the complete range of *Neocallimastigales* axenic cultures used in this study demonstrated that within individual cultures the ITS1 region was polymorphic in size, giving variable numbers of peaks with different relative intensities (see supporting information, Fig. S1). Pearson- and Dice-based cluster analyses of the culture profiles generated four consistent and distinct clusters (Pearson-based analysis is shown in Fig. 1). Comparison between the genus affiliation of the culture (Table 1) and the ARISA clusters resulted in no obvious correlation. Amplification of rumen content generated a complex ARISA profile (Fig. 2), with sequence analysis (EU414760–EU414770) confirming the specificity of the primers for *Neocallimastigales* (Table S1).

Application of the *Neocallimastigales* probe-based QPCR assay showed that detection and quantification was linear over the range examined (0.006–2400 pg of *Neocallimastigales* DNA per well) with the linear model accounting

for 99.7% of the data. PCR efficiency was reproducibly > 92%, with an average efficiency of 98% ( $n=9$ ). No detectable signal was observed with perennial ryegrass alone.

### ARISA of rumen-incubated fresh perennial ryegrass

Anaerobic fungal colonization of damaged fresh perennial ryegrass incubated in the rumen was assessed by ARISA. Profiles for the damaged fresh perennial ryegrass (0 min) gave no peaks reliably above the background (Fig. 3). However, within 5 min of ruminal incubation, distinct peaks associated with anaerobic fungi colonizing the damaged fresh perennial ryegrass were evident. With both cows, an increase in the intensity of the peaks was observed with time, with minor peaks in early time-point samples becoming more pronounced relative to the two dominant peaks (366 and 374 bases). Pearson-based cluster analysis of the ARISA



**Fig. 1.** Pearson cluster analysis of ARISA profiles from *Neocallimastigales* axenic cultures. Dendrogram branches are labelled with the strain (see Table 1 for further details of the cultures) and distinct clusters are highlighted as groups i–iv. Band intensity and position represent the ARISA peak intensity and corresponding fragment size. Fragments ranged in size from 355 bases (Isol1) to 441 bases (W7).

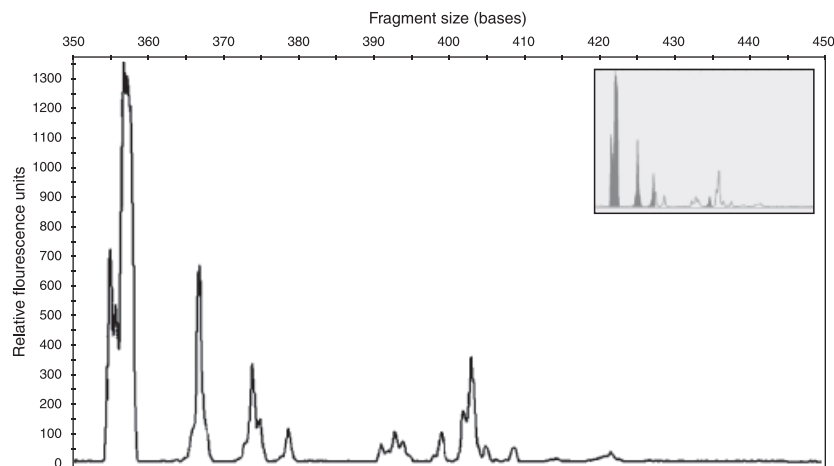
profiles from both cows demonstrated that the ARISA profiles showed > 92% similarity, with the exception of two outlying samples (5 and 10 min machine washed) from cow B (Fig. 4). No clustering in relation to cow, time or washing procedure was observed, although with cow A, the 10- and 15-min samples and 5- and 30-min samples were located in two different subclusters. Clusters generated using Dice differed from those observed by Pearson, with all the 5-min samples from cow B forming an outlying cluster and a tendency for the remaining samples to cluster by animal (Fig. S2).

### QPCR analysis of rumen-incubated fresh perennial ryegrass

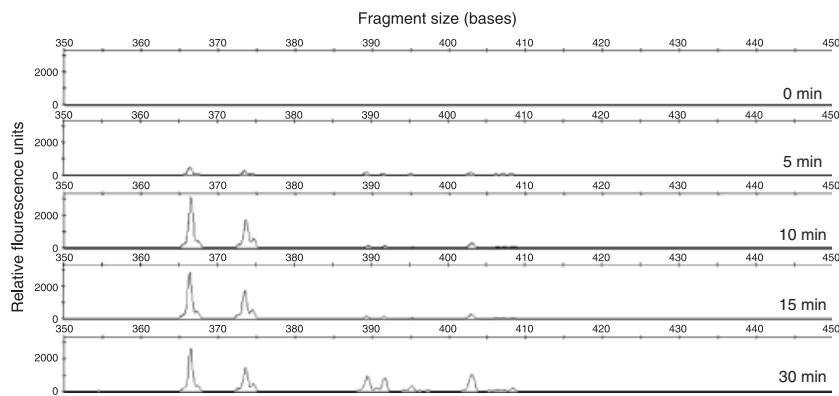
Quantification of the anaerobic fungi colonizing the damaged fresh perennial ryegrass was carried out using the *Neocallimastigales* probe-based QPCR assay. The exponential model fitted to the QPCR data accounted for 96.5% of the variation in the data. There was an increase in the amount of *Neocallimastigales* DNA with increasing incubation time ( $P=0.01$ ) (Fig. 5). There was no evidence to suggest that the quantity of *Neocallimastigales* DNA ( $P=0.44$ ) or the fractional rate of increase in quantity ( $P=0.12$ ) differed between washing treatments nor was there any interaction between washing treatment and incubation time ( $P=0.28$ ). Assuming an overall model, the lag period was estimated to be 3.7 min (SE 1.7) before *Neocallimastigales* DNA was detected on fresh forage, and the fractional rate of increase in the amount of *Neocallimastigales* DNA was 0.032 (SE 0.030)  $\text{min}^{-1}$ .

### Discussion

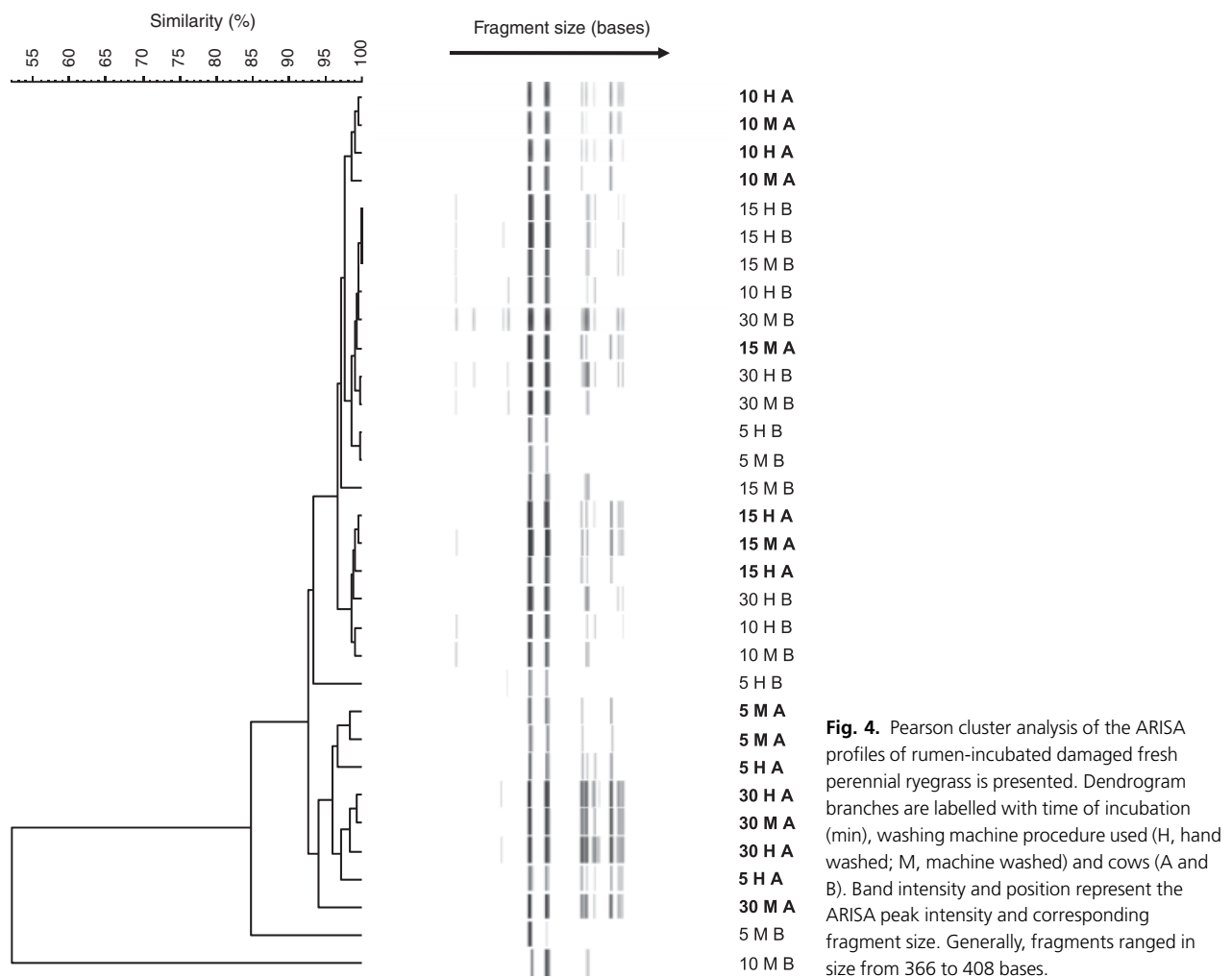
This study combined the molecular analysis of both the *Neocallimastigales* population composition and size in order to characterize the anaerobic fungal initial colonization



**Fig. 2.** *Neocallimastigales* ARISA profile of rumen content. A sample of rumen content from a nonlactating Holstein–Friesian dairy cow fed a barley straw diet was profiled, and the corresponding PCR product was cloned and sequenced. The cloned sequences that matched fragment sizes within the profile are highlighted within the inset (black fill).



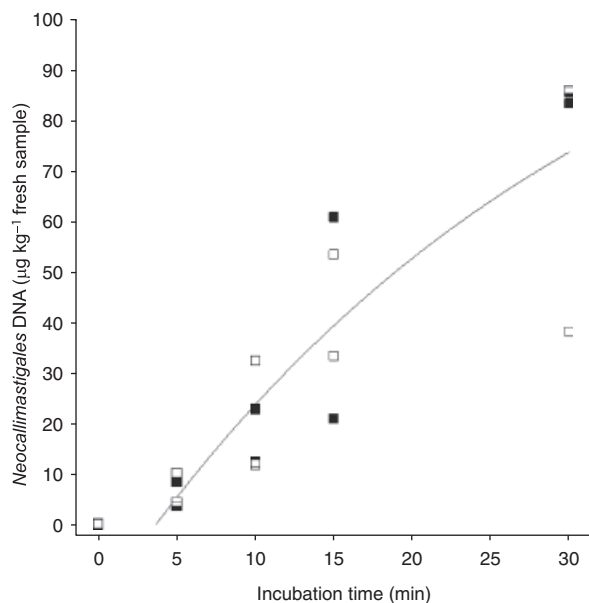
**Fig. 3.** ARISA profiles of damaged fresh perennial ryegrass before (0 min) and after (5–30 min) ruminal incubation *in sacco*. Profiles shown are representative of the samples generated with both hand and machine wash procedures for cow A.



**Fig. 4.** Pearson cluster analysis of the ARISA profiles of rumen-incubated damaged fresh perennial ryegrass is presented. Dendrogram branches are labelled with time of incubation (min), washing machine procedure used (H, hand washed; M, machine washed) and cows (A and B). Band intensity and position represent the ARISA peak intensity and corresponding fragment size. Generally, fragments ranged in size from 366 to 408 bases.

events on fresh forage entering the rumen. Anaerobic fungi from the ruminant gut have been investigated previously using molecular-based methods (Brookman & Nicholson, 2005; Denman & McSweeney, 2006). However, *in silico*

analysis and generation of new sequence data in this study confirmed that the area of the ITS1 region targeted previously by published molecular-based methods (Brookman & Nicholson, 2005; Denman & McSweeney, 2006) was



**Fig. 5.** Quantification of the *Neocallimastigales* population colonizing fresh perennial ryegrass after rumen incubation. The amount of *Neocallimastigales* DNA extracted from damaged fresh perennial ryegrass incubated *in sacco* in the rumen was determined for samples that were processed by machine (■) or hand washing (□). Data points represent the means of two replicate polyester bags from the same cow.

not conserved in more recently generated sequence data (i.e. *Anaeromyces* sp. GE09). In order to ensure that the analysis of anaerobic fungi was comprehensive and specific in this study, new primers and a probe were developed and validated by *in silico* analysis, sequencing and QPCR.

The clusters of ARISA profiles generated from the limited number of cultures used in this study did not obviously correlate with the genus assignment of the axenic fungal cultures. This is in line with the previous observation that the length variation of the ribosomal intergenic spacer regions, which although sufficiently robust to monitor *Neocallimastigales* populations, only has an approximate relationship with genus (Brookman & Nicholson, 2005). The sequence of the ITS1 region, however, has been demonstrated to be effective in enabling differentiation of anaerobic fungi to presumptive genera (Brookman *et al.*, 2000; Tuckwell *et al.*, 2005).

Application of the ARISA methodology to rumen-incubated damaged fresh perennial ryegrass demonstrated that anaerobic fungi attached to plant particles within 5 min. Cluster analysis of the ARISA profiles indicated that no consistent change in the population composition of the anaerobic fungi occurred with respect to time and/or wash treatment in the two animals studied. These results are consistent with the corresponding rumen bacterial population composition, which also showed no obvious change with respect to these factors in a 30-min timescale (Edwards

*et al.*, 2007). Because of the 30-min time-scale used in this study, the anaerobic fungi detected are likely to represent attached, encysted and potentially germinated zoospores. Previous studies have observed that in animals fed daily, the timing of peak zoospore densities in rumen fluid depends on the genus, with *Piromyces* and *Caecomyces* zoospores peaking 1 h after feeding (Orpin, 1976, 1977c) and *Neocallimastix* after *c.* 30 min (Orpin, 1974, 1975, 1976). How these findings correlate to grazing animals remains unclear, as in free-ranging animals, zoospores may be seen in rumen contents at any time of day (Orpin, 1994). In this study, the ARISA fragment sizes found to dominate the colonizing fungal population were 366 and 374 bases in size, which corresponds to the size range represented by the cluster of axenic cultures belonging to the polycentric genera *Anaeromyces* and *Orpinomyces*. Information on the timing of peak zoospore densities after feeding from either of these two genera is not currently available.

QPCR-based analysis of the rumen-incubated, damaged fresh perennial ryegrass demonstrated that there was a delay in the onset of attachment/encystment by anaerobic fungi, followed by a continued increase in population size. There was no evidence of the population size stabilizing within the 30-min incubation period. This dynamic contrasted with that of initial colonizing rumen bacteria, which exhibited no lag and appeared to stabilize after 15 min (Edwards *et al.*, 2007). Furthermore, the tendency for population sizes of colonizing bacteria to be lower in machine-washed samples (Edwards *et al.*, 2007) was not observed with colonizing anaerobic fungi. Although it has been reported previously that the use of *in sacco* methods has potential limitations, particularly in relation to pore size restricting a representative influx of ruminal microorganisms, careful selection of bag pore size and standardization of procedures can limit the majority of these issues (Meyer & Mackie, 1986; Vanzant *et al.*, 1998). As it has been demonstrated that a minimum pore size of 30 µm is needed to allow maximal influx of microorganisms the size of small protozoa (16–35 µm) or smaller (i.e. bacteria) (Meyer & Mackie, 1986), it is assumed that the observed differences in fungal and bacterial colonization are not due to differential influx. Fundamental differences in the biology of bacteria and anaerobic fungi, and their attachment mechanisms, therefore are considered a more likely explanation.

The consistent presence of zoospores in free-ranging animals suggests that the observed lag in colonization of forage by anaerobic fungi is unlikely to be a delay associated with plant-induced zoosporogenesis (Orpin, 1977b; Orpin & Greenwood, 1986). As zoospores locate newly ingested plant material in response to chemoattractants, such as soluble sugars, the observed lag may be associated with a delay in their detection. Orpin & Bountiff (1978) demonstrated *in vitro* that chemotaxis of *N. frontalis* zoospores to

glucose (1 mM) was delayed by 5 min. After this delay, the rate of accumulation of zoospores increased to a constant maximum between 5 and 30 min, before decreasing rapidly. Lower concentrations of glucose decreased the maximum rate of accumulation of zoospores. The glucose content of perennial ryegrass leaves has been shown *in vitro* to be sufficient to elicit chemotaxis, encystment and germination of zoospores within 30 min (Orpin & Bountiff, 1978). Based on this, and the findings in this study, it is suggested that the dynamics of initial colonization of fresh plant material by anaerobic fungi in grazing animals is primarily mediated by the time taken for anaerobic fungi to locate, attach and encyst on plant material in the rumen.

Compared with anaerobic fungi, colonizing rumen bacteria are more likely to rapidly utilize soluble sugars from freshly ingested plant material, particularly because substantial bacterial colonization of damaged fresh perennial ryegrass occurs within 5 min (Edwards *et al.*, 2007). In addition to decreasing the rate of zoospore accumulation, lower glucose concentrations also result in the time taken for 100% germination to be delayed for up to 6 h (Orpin & Bountiff, 1978). Therefore, rumen bacteria may not only influence zoospore chemotaxis but also encystment and subsequent fungal life cycle stages, particularly with diets low in complex lignocellulosic plant material.

In conclusion, the dynamics of initial colonization of fresh forage by anaerobic fungi in grazing animals differs substantially from that of rumen bacteria, with fungal dynamics suggested to be primarily mediated by the time taken for fungal zoospores to locate, attach and encyst on plant material. Further research is needed to assess what factors can modify subsequent stages of the fungal life cycle *in vivo*. This information is central to the future delivery of more sustainable forage-based livestock production systems.

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## Supporting Information

Additional Supporting Information may be found in the online version of the article.

**Table S1.** Sequence analysis of cloned rumen content ARISA amplicons.

**Fig. S1.** ARISA profiles generated from axenic cultures of *Neocallimastigales*.

**Fig. S2.** Dice cluster analysis of the ARISA profiles of rumen incubated damaged fresh perennial ryegrass.

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