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Development and use of quantitative competitive PCR assays for relative quantifying rumen anaerobic fungal populations in both *in vitro* and *in vivo* systems

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ABSTRACT

This paper describes the use of a quantitative competitive polymerase chain reaction (QC-PCR) assay; using PCR primers to the rRNA locus of rumen fungi and a standard-control DNA including design and validation. In order to test the efficiency of this method for quantifying anaerobic rumen fungi, it has been attempted to evaluate this method in *in vitro* conditions by comparing with an assay based on measuring cell wall chitin. The changes in fungal growth have been studied when they are grown in *in vitro* on either untreated (US) or sodium hydroxide treated wheat straw (TS). Results showed that rumen fungi growth was significantly higher in treated samples compared with untreated during the 12 d incubation ($P < 0.05$) and plotting the chitin assay's results against the competitive PCR's showed high positive correlation ($R^2 \geq 0.87$). The low mean values of the coefficients of variance in repeatability in the QC-PCR method against the chitin assay demonstrated more reliability of this new approach. And finally, the efficiency of this method was investigated in *in vivo* conditions. Samples of rumen fluid were collected from four fistulated Holstein steers which were fed four different diets (basal diet, high starch, high sucrose and starch plus sucrose) in rotation. The results of QC-PCR showed that addition of these non-structural carbohydrates to the basal diets caused a significant decrease in rumen anaerobic fungi biomass. The QC-PCR method appears to be a reliable and can be used for rumen samples.

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Introduction

The anaerobic fungi are now known to be one of the most significant groups of rumen microorganisms (Trinci *et al.* 1994; Orpin *et al.* 1997) and are primary colonizers of fibrous plant material that are able to degrade lignin-containing plant cell walls (Bauchop 1979; Akin *et al.* 1990). Since the discovery of anaerobic rumen fungi by Orpin (1975), considerable effort has been directed towards documenting the effects of diet changes, times of feeding and feeding frequency on their populations within the rumen (Dehority & Tirabasso 2001; Rezaeian *et al.* 2005; Denman & McSweeney 2006). Early work on the fungi by Bauchop (1979), based on zoospore abundance indicated that diet had a substantial effect on fungal

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populations. Rezaeian *et al.* (2005), showed sodium hydroxide treated barley straw caused to increased rumen fungi in *in vitro* culture and it has been proposed that diets rich in soluble carbohydrates inhibited the production of zoospores in the rumen (Grenet *et al.* 1989; Roger *et al.* 1990; Kamra *et al.* 2003).

Direct enumeration and marker techniques have been developed for the estimation of fungal populations in the rumen. Zoospore counts could not be used to estimate fungal biomass because they do not take into account the thallus stages that produce them (Orpin 1994). Furthermore counting zoospores is both time consuming and difficult (Denman & McSweeney 2006). Fungal growth was also assessed by biochemical markers such as the measurement of phospholipids (Orpin & Letcher 1979) and the amount of hydrogen or formic acid produced by the culture (Lowe *et al.* 1987; Mountfort & Asher 1985). However, these assays are frequently interfered with by chemicals in the growth media and rumen fluid and therefore cannot be used to accurately estimate the growth of ruminal fungi (Orpin & Letcher 1979). Chitin as a component of the cell wall of anaerobic fungi has also been used as a marker of fungal populations (Phillips & Gordon 1989; Rezaeian *et al.* 2004). However the chitin content of rumen fungal cell walls may vary according to the species, their age and conditions of growth (Gay 1991).

With recent advances in molecular enumeration methods, in particular 18S rDNA gene probing methods, researchers were able to monitor fungal species within the rumen (Stahl *et al.* 1988). However due to the high level of conservation within the fungal 18S DNA gene sequence (Bowman *et al.* 1992), the more variable internal transcribed space 1 region (ITS1) provides a more appropriate gene sequence for species identification. Despite the recent introduction of real-time PCR method for the rapid quantification of the target DNA sequences (Freeman *et al.* 1999), the use of the quantitative competitive PCR (QC-PCR) technique continues to play an important role in nucleic acid quantification because it is more cost effective (Franz *et al.* 2001). The procedure relies on the co-amplification of the sequence of interest with a serially diluted synthetic DNA fragment of known concentration (competitor), using a single set primers (Gaiger *et al.* 1995; Lion *et al.* 1992). In rumen microbial ecosystem studies, this method has so far only been used to quantify rumen bacteria. This study describes the development of QC-PCR assays, and assesses its validity for relatively quantifying rumen fungi populations in both *in vitro* and *in vivo* systems.

Materials and methods

Validation of quantitative competitive PCR assays

Sample preparation

Samples of wheat straw were chopped (2–5 cm) and 160 g of chopped samples were soaked in 3 L of NaOH solution (10 % w/v) in a closed plastic bag for 80 h. Treated straw (TS) was washed under tap water until the yellow colour resulting from the NaOH treatment was eliminated. The same amount of chopped samples were also washed under tap water for using as the control (untreated, US). Then treated and untreated straw samples were dried using air forced oven dry (95 °C, 24 h), weighed and milled (1 mm screen) (Rezaeian *et al.* 2005). Wheat straw was used as the carbon source for growth of the rumen anaerobic fungi in *in vitro* system.

Isolation and culturing of rumen anaerobic fungi

Rumen fungi were isolated from the wheat straw incubated in the rumen of a fistulated steer (Rezaeian 1996) and grown using the procedures described by Joblin (1981) under anaerobic conditions at 39 °C for 3 d. These isolates were then used as a source of inoculum for further experimental studies. Serum bottles containing 90 ml of fungi culture medium and 1 g of treated or untreated wheat straw were used to culture the isolated fungi at 39 °C. Sub-culturing was done three times to obtain pure cultures. The identification of these pure fungal cultures was confirmed using specific anaerobic rumen fungal primers.

Measurement of chitin

150 mg of the air-dried samples from *in vitro* cultures were hydrolyzed with 6 ml of 6 N HCl for 4 h in 105 °C. After cooling, the hydrolysate was centrifuged at 3200 rpm for 30 min at 4 °C. Then, supernatant was filtered using a 0.45 mm filter and freeze dried. The chitin contents of each sample were determined from the glucosamine hydrochloride equivalent resulting from hydrolysis as described by Chen & Johnson (1983).

Comparison of fungal biomass of different diets by quantitative competitive PCR assays

Rumen sampling

Samples of rumen fluid and digesta were collected from four fistulated Holstein steers (live weight = 250 ± 18 kg) which were fed four diets in a 4 × 4 Latin square design with 21 d periods; 17 d diet adjustment and 4 d sample collection. The basal diet was formulated to contain alfalfa hay, barely grain, soybean meal and sugar beet pulp (400, 290, 190 and 50 g kg^{-1}, respectively). Starch (St) or sucrose (Su) or a 1:1 mixture of starch and sucrose (St + Su) was added to the control diet at the rate of 70 g kg^{-1} DM. Diets were offered as 2–2.5 times of maintenance requirements (7 kg DM/d). Details of treatment are given in Table 1. Samples were taken at the end of each period just 6 h after feeding, from the central portion of the rumen with initial course filtration through an insect screen with a medium mesh size (2 × 1.5 mm) (Denman & McSweeney 2006). Rumen pH was measured directly (691 pH meter) and rumen ammonia–N was determined by steam distillation (Kjeltec Auto, 1300, Foss Electric, Copenhagen, Denmark). The pooled filtrate which was used for DNA extraction, in excess of 200 mL per sampling, contained digesta plant particles and rumen fluid.

DNA extraction

Total genomic DNA was isolated using Guanidine Thiocyanate–Silica Gel method (Boom *et al.* 1990). For rumen samples, 0.5 mL aliquot was taken from the 200 mL sample using a wide-bore pipette, so as to ensure that a homogeneous sample containing plant particles and liquid was obtained. For pure cultures, genomic DNA was extracted from biomass

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