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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

Mohammad H. SEKHAVATI[a,*], *Mohsen Danesh MESGARAN*[a], *Mohammad R. NASSIRI*[a], *Tahereh MOHAMMADABADI*[b], *Farkhondeh REZAII*[a], *Adham FANI MALEKI*[a]

[a]*Department of Animal Science, Excellence Center for Animal Science, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad 098, Iran*

[b]*Department of Animal Science, Ramin Agriculture and Natural Resource University, Molasani, Ahvaz, Iran*

ARTICLE INFO

Article history:
Received 27 October 2007
Received in revised form
23 June 2009
Accepted 20 July 2009
Available online 30 July 2009
Corresponding Editor:
Gordon W. Beakes

Keywords:
Chitin
Non-structural carbohydrate
QC-PCR
Rumen anaerobic fungi

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.

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

* Corresponding author. Tel.: +98 9155077543.
E-mail address: hadisekhavati@gmail.com
0953-7562/$ – see front matter
doi:10.1016/j.mycres.2009.07.017

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

Table 1 – Composition of the experimental diets (%) fed to four rumen fistulated steers

Item	Treatments[a]			
	Basal diet	St	Su	St + Su
Alfalfa (%)	40	40	40	40
Concentrate (%)	60	60	60	60
Barley	36	29	29	29
Soybean meal	19	19	19	19
Beet pulp	5	5	5	5
Starch	–	7	–	3.5
Sucrose	–	–	7	3.5

St: Starch as 70 mg g^{-1} DM.
Su: Sucrose as 70 mg g^{-1} DM.
St + Su: Starch as 35 mg g^{-1} DM and sucrose as 35 mg DM^{-1}.
a Basal diet: alfalfa hay, barely grain, soybean meal and sugar beet pulp (400, 290, 190 and 50 g kg^{-1}, respectively).

harvested by centrifugation (2000 rpm, 10 min) from broth medium (Fliegerova *et al.* 2006).

PCR primers and amplification

The general anaerobic fungal primers (GAF) previously designed by Denman & McSweeney (2006) from multiple alignments of fungal 18S ribosomal and ITS1 gene sequences were used in this study and are listed in Table 2. PCR amplification of rumen anaerobic fungi DNA produces a 110-bp product when amplified with the universal primers. The PCR mixture contained 50 ng of template DNA, 2 μl 10× PCR buffer, 2.5 mM $MgCl_2$, 200 μM each dNTPs, 10 pM of each primer and 1 U Taq DNA polymerase. The PCR was performed in a final volume of 25 μl sealed in a capillary tip, and thermocycling was carried out in a model 2000 (Biometra). The PCR amplification condition was as follows: denaturation at 94 °C for 4 min followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. The PCR products were analyzed by running on 2 % agarose gels containing ethidium bromide, and visualized for a single specific band and the absence of primer dimer products by UV trans-illumination.

Construction of the standard control

The non-homologous competitor (standard control) was designed to contain GAF-universal primer binding sequence of ~25 bp at ends encompassing a phage sequence (phage lambda sequence location 46 775–46 797 and 46 891–46 912, respectively, according to GenBank accession no. NC_1416). The standard control was generated by amplification of the enterobacteria phage lambda DNA with hybrid primers (Fig 1). The hybrid primers are listed in Table 2. The PCR was performed in a final volume of 25 μl sealed in a capillary tip, and thermocycling was carried out in a model 2000 (Biometra). The PCR amplification conditions were as follows: denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min followed by a final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis in agarose gels, stained with ethidium bromide, and visualized by UV trans-illumination.

Table 2 – PCR primers for amplifying target and non-homologous competitors

Target species
Anaerobic rumen fungi
GAF_1 5′-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3′
GAF_2 5′-CAAATTCACAAAGGGTAGGATGATTT-3′
Enterobacteria phage lambda
$LaGAF_1$ 5′-GAGGAAGTAAAAGTCGTAACAAGGTTTC[a]GAA GTTCGCAGAATCGTATGTG-3′
$LaGAF_2$ 5′-CAAATTCACAAAGGGTAGGATGATTT[a]GCTG TGGACATAGTTAATCCG-3′

a The 5′ ends of hybrid primers contained a GAF-universal sequence.

Quantification of PCR products

The PCR products were quantified by photographing agarose gels with Polaroid 665 film (Polaroid, St. Albans, England), which produce a negative image of the photograph. The negative was scanned with a GS-670 image densitometer (Bio-Rad, Hercules, California, USA) and analyzed with ImageJ 1.38x software (National Institutes of Health, USA). To correct for differences in the fluorescence of ethidium bromide-stained PCR fragments of different size (Piatak *et al.* 1993), the intensities of amplified standard control was multiplied by the ratio 110/191.

Statistical analysis

Statistical analyses of the data for evaluation of the effect of treatments on the changes of fungal populations, rumen pH and ammonia–N were performed by ANCOVA (analysis of covariance) and ANOVA using the GLM procedure of SAS. Since the intensity of amplified target bands is linearly related to the intensity of standard-control bands, for increasing precision, ANCOVA were performed for these data and the intensity of standard-control bands used as a co-variable (Snedecor & Cochran 1967). Plotting the chitin assay's results against the QC-PCR's was evaluated by simple regression by using JMP software (ver 7.0, SAS Institute Inc., USA). Duncan test was carried out at the 5 % ($P < 0.05$) and 1 % ($P < 0.01$) level to determine the statistical significance of differences between treatments.

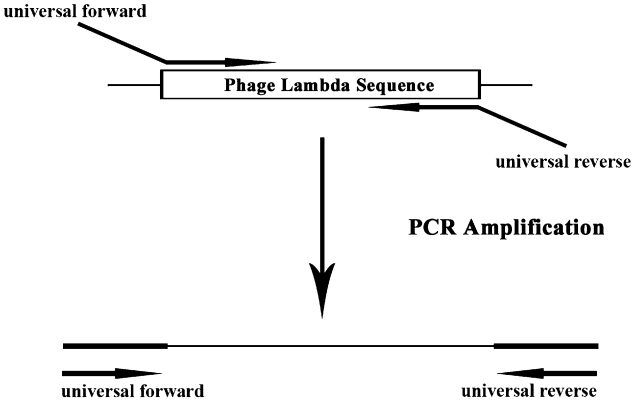

Fig 1 – Construction of non-homologous competitor with hybrid primers. The PCR product (191-bp) was purified and used as the standard control.

Results

Validation of quantitative competitive PCR assays

PCR amplification using the anaerobic fungal primers (GAF) produced fungal-specific amplicons (Fig 2) of the expected (110 bp) size found by Denman & McSweeney (2006). PCR amplification of the enterobacteria phage lambda DNA using overhang primers (LaGAF) produced the expected size (191 bp). The relative amplification efficiencies of target and standard-control DNA were determined from a plot of the log ratio of intensities of the amplified target DNA to standard control against the concentration of standard-control DNA. Co-amplification of DNA from pure culture (55 ng μl^{-1}), with dilutions of the standard control (Fig 2) resulted in a line with a slope 0.868 and a high positive correlation ($R^2 = 0.97$). This indicated that the target and control standard DNA were amplified with an appropriate equivalent efficiency. The line which intersects the x axis at 10^{-4} (Fig 2), indicated that this dilution of the standard control was optimal for the detection of 55 ng μl^{-1} DNA of the pure culture.

In order to compare the accuracy of chitin assay and QC-PCR method, the inter-assay precision was determined by computing the coefficients of variance (CV) of the obtained data for each method (Table 3). Determination of variation was done in 1 ml culture medium samples in four replicates per each treatment for chitin measurements and DNA extraction. Using both QC-PCR and chitin assays it was shown that growth of rumen fungi was significantly ($P < 0.05$) higher in sodium hydroxide treated samples compared with untreated straw during the 12-d incubation period (Table 3). There

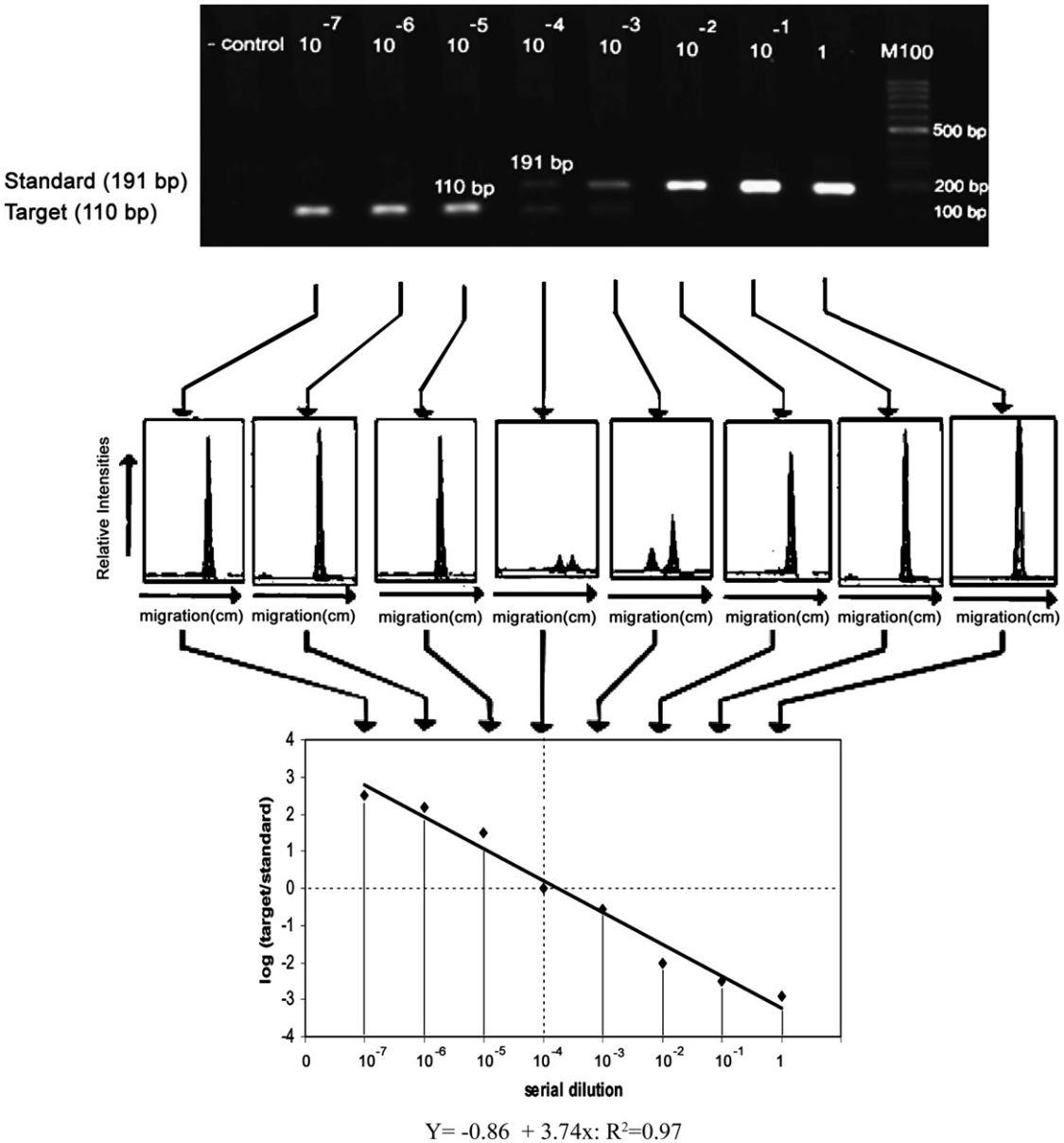

Fig 2 – Standard-control amplification efficiency. Dilutions of standard control were coamplified with DNA from pure culture, and the log ratio of intensities of amplified target DNA to standard was plotted against the dilutions of the standard control.

Table 3 – Changes in chitin content (mg ml^{-1} of culture medium) and the log ratio of intensities of amplified standard control to target DNA (arbitrary unit) during 12 d growth of rumen anaerobic fungi on untreated (US) and sodium hydroxide treated (TS) straw

Method	Treatment	Mean	SE	CV
Chitin assay	TS	6.622[a]	0.511	18.90 %
	US	4.888[b]	0.210	10.90 %
Competitive PCR	TS	0.133[c]	0.185	3.40 %
	US	0.082[d]	0.163	4.85 %

Values are mean of four replicates means in the same row with different superscripts differ significantly ($P < 0.05$) for each item; SE: standard error of mean.

were high correlations between the levels of cell wall chitin that were assessed by chitin assay with competitive PCR's results for both untreated (Fig 3a) and sodium hydroxide treated straw (Fig 3b).

Comparison of fungal biomass, pH and ammonia–N concentration in different diets

The results of using QC-PCR to assess the effects of changing the diet on the biomass of rumen fungal populations *in situ* is summarized in the histograms in Fig 4a. This data is also correlated with both levels of ammonia and pH (Fig 4b,c). Diets containing non-structural carbohydrate all decreased rumen fungal populations in comparison with those found on animals fed the basal diet (Fig 4). Fungi biomass was the lowest for diet containing the mixture of starch and sucrose ($P < 0.05$). Ammonia–N concentration was lower when steers were fed on St or Su rather than on the basal diet ($P < 0.05$), although there was no significant differences in pH between any of the experimental diets (Fig 4).

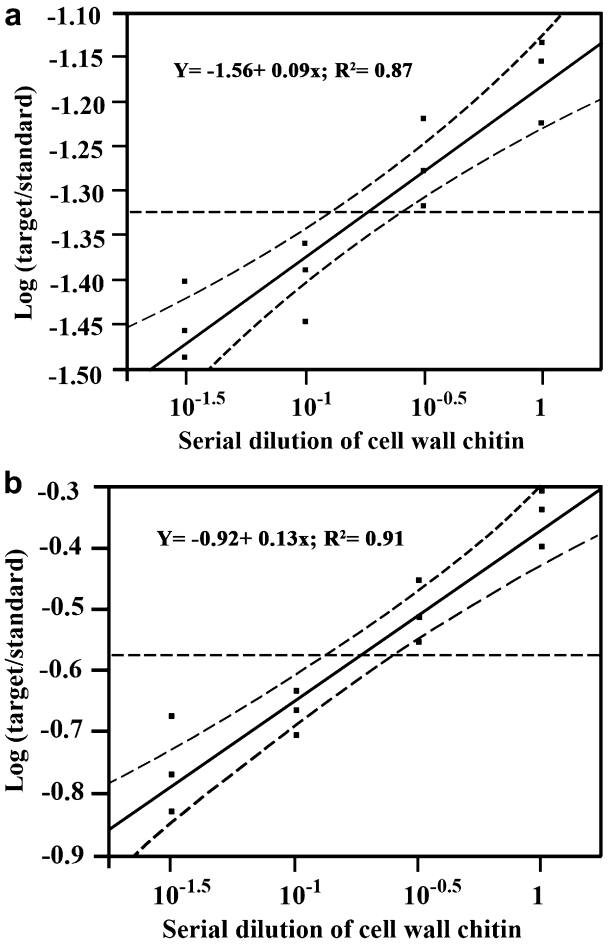

Fig 3 – Relationship between chitin content (mg ml^{-1}) and competitive PCR's results (arbitrary unit) in the culture medium containing untreated (a) and NaOH treated (b) wheat straw during 12 d growth of rumen anaerobic fungi. Cell wall chitin content for 1, $10^{-0.5}$, 10^{-1} and $10^{-1.5}$ of serial dilution were contained 4.42, 0.88, 0.176, 0.035 and 6.32, 1.26, 0.25, 0.05 mg ml^{-1}, for untreated and treated wheat straw respectively. In each dilution 3 signal bands were used for analyzing the log ratio of intensities of amplified target DNA to standard.

Discussion

Anaerobic rumen filamentous fungi form extensive, often intramatrical, rhizoidal systems which makes accurate enumeration using a microscopic impractical (Dehority & Orpin 1997). Enumeration of anaerobic fungi as thallus-forming units (TFU) is an end-point dilution procedure based on the most probable number (MPN) technique. The MPN method developed by Theodorou *et al.* (1990) and Obispo & Dehority (1992) consistently yielded higher fungal counts when compared with direct microscopic counts (Dehority & Orpin 1997). One serious problem with these methods is that the rumen fungi often grow as a nonhomogenous culture and cannot be accurately serially diluted. Another approach taken to assessing rumen fungal populations is to assay biochemical markers, such as chitin assay was used for assessing rumen fungal biomass (Orpin & Joblin 1988; Rezaeian *et al.* 2004). However, the likely interference of glucosamine present in bacterial cell walls with this assay (Lehninger *et al.* 1993) and the possible contamination with aerobic fungi (Orpin & Greenwood 1986) may affect the efficacy of this method.

Real-time PCR method has recently become established as a standard protocol to obtain quantitative PCR data. However, it is a relatively expensive technique. Consequently we have attempted to evaluate the use of the much more cost-effective quantitative competitive PCR technique (QC-PCR) in the evaluation of rumen fungal populations using non-specific primers designed for anaerobic rumen fungi by Denman & McSweeney (2006). In an *in vitro* system we evaluated the efficacy of this technique on either untreated or sodium hydroxide treated of wheat straw over a 12-d period as described by Rezaeian *et al.* (2004) and directly compared the data with estimates of fungal biomass using the chitin bioassay. The results confirmed the findings of Rezaeian *et al.* (2004) that straw treated with sodium hydroxide supported a higher biomass of anaerobic rumen fungi. There was a high positive correlation ($R^2 \geq 0.87$) between the results obtained with the two methods. Furthermore the low mean values of CV (Table 3) in

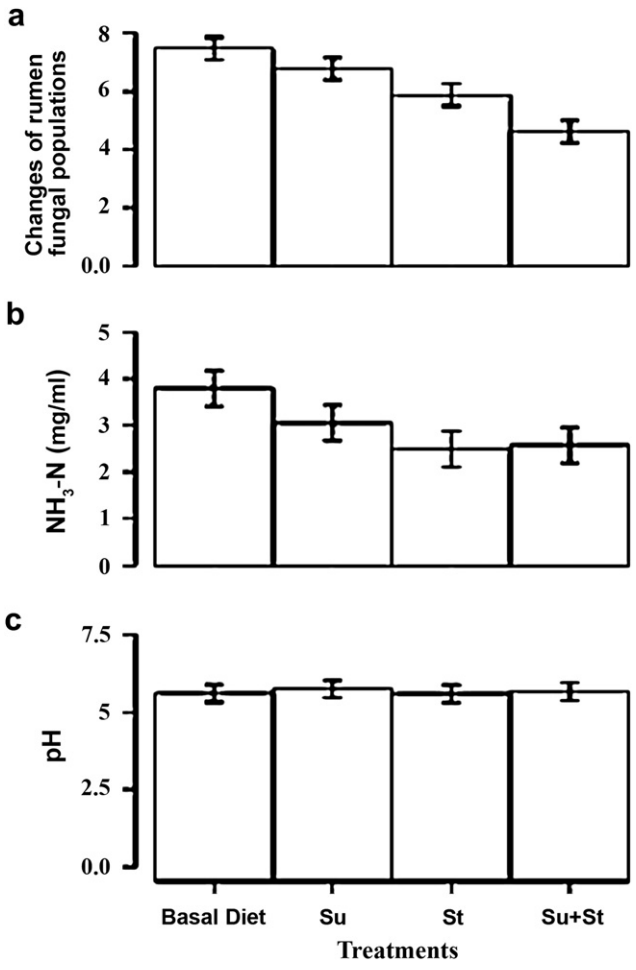

Fig 4 – Quantitative changes of rumen anaerobic fungal populations (the mean of intensity of amplified target bands for each treatment (arbitrary unit)), rumen pH and ammonia–N concentration (mg ml^{-1}) in steers fed with different diets (basal diet: alfalfa hay, barely grain, soybean meal and sugar beet pulp (400, 290, 190 and 50 g kg^{-1}, respectively), St: Starch as 70 mg g^{-1} DM, Su: Sucrose as 70 mg g^{-1} DM, St + Su: Starch as 35 mg g^{-1} DM and sucrose as 35 mg DM^{-1}).

repeatability (CV = 3.40 % and 4.85 % for ST and UT, respectively) in the QC-PCR method compared with the chitin assay protocol (CV = 18.90 % and 10.90 % for ST and UT, respectively) indicates that the molecular method is more reliable than the chitin assay for evaluation of changes in fungal biomass. Appropriate slope and high positive correlation ($R^2 = 0.97$) of DNA from pure culture with dilutions of the standard control indicated that standard control and target DNA amplified with equivalent efficiency in the same reaction. Furthermore, it showed that the synthetic standard control was suitable for the QC-PCR reaction (Fig 2). PCR amplification of rumen microbial DNA using the anaerobic fungal primers (GAF) designed by Denman & McSweeney (2006) produced fungal-specific amplicons of the expected size (110 bp). Using these primers for evaluating the changes of anaerobic fungal biomass in this molecular approach can be one of the advantage of this method versus the chitin assay, because these specific primers cannot amplify contaminants of non ruminant fungal DNA (Denman & McSweeney 2006) that may exist in the samples or the animal diet, whereas these contaminates may be detected with the chitin assay (Orpin & Greenwood 1986). However, *in silico* analysis and generation of new sequence data that where obtained by Edwards *et al.* (2008) confirmed that the area of the ITS1 region which had been targeted previously by published molecular-based methods (Brookman & Nicholson 2005; Denman & McSweeney 2006) was not conserved in more recently generated sequence data (i.e. *Anaeromyces* sp. GE09). Therefore the current primers may not be detecting all rumen anaerobic species.

The use of conventional and QC-PCR is to some extent restricted by the presence of PCR inhibitors that maybe exist in rumen fluids (Degraves *et al.* 2003; Harms *et al.* 2003; Klaschik *et al.* 2002; Tichopal *et al.* 2004). These inhibitors can dramatically reduce the sensitivity and amplification of PCR (Dieffenbach & Dveksler 2003). However the plot of log ratio of intensities of amplified target and standard-control DNA per sample of each treatment in *in vivo* conditions to dilutions of the standard control suggest that the efficiency of QC-PCR was not influenced by inhibitors in these experiments (Table 4).

Under our *in vivo* conditions study, the results of QC-PCR showed that the rumen fungal population was decreased by diets containing non-structural carbohydrates compared with the basal diet ($P < 0.05$) (Fig 4). The lowest overall fungal detection was in diets supplemented with a mixture of starch plus sucrose that followed by diets containing starch and sucrose, respectively (Fig 4). So, this decrease in measured fungal biomass was likely due to these carbohydrates. The results of this study are in agreement with previous results that indicated diets rich in soluble carbohydrates resulted in rapid fermentation and inhibited the production of zoospores in the rumen (Grenet *et al.* 1989; Roger *et al.* 1990; Kamra *et al.* 2003). But, the present results indicated that the presence of non-structural carbohydrate diets did not alter ruminal pH 6 h after feeding (Fig 4). Khezri *et al.* (2009) demonstrated that the rumen pH was not affected as sucrose replaced starch (7.5 % of diet DM) in dairy cow diet. This is in agreement with the conclusions drawn by Mould *et al.* (1983) who conducted

Table 4 – Appropriate mean slopes and high positive correlations which obtained with plotting the log ratio of intensities of amplified target and standard-control DNA for per sample of each treatment to dilutions of the standard control (values are mean of four replicates in each treatment)

Treatment[a]	Slope	R^2
Basal diet	0.85 ± 0.08	0.96 ± 0.01
Su	0.88 ± 0.05	0.95 ± 0.01
St	0.89 ± 0.09	0.97 ± 0.01
St + Su	0.78 ± 0.07	0.98 ± 0.01

St: Starch as 70 mg ml^{-1} DM.
Su: Sucrose as 70 mg ml^{-1} DM.
St + Su: Starch as 35 mg ml^{-1} DM and sucrose as 35 mg DM^{-1}.
a Basal diet: alfalfa hay, barely grain, soybean meal and sugar beet pulp (400, 290, 190 and 50 g kg^{-1}, respectively).

an *in vivo* study to evaluate the effect on grass hay digestion of incrementally increasing grain in the diet while maintaining ruminal pH by infusing bicarbonate. In their experiment, they observed that the depression in the extent of grass hay digestion was substantially, although not completely, overcome by maintaining ruminal pH. The inability to completely alleviate the impact on NDF (neutral detergent fiber) digestion was attributed to what they called a ''carbohydrate effect''. It is thought that the effect that the presence of non-structural carbohydrate has on fiber digestion, which is almost entirely carried out by rumen fungi (Akin *et al.* 1990), is due to the direct effect of these substrates in decreasing the rumen fungal population. One suggestion proposed to explain the effect of non-structural carbohydrates on decreasing fiber digestion is that cellulolytic microbes are unable to compete for essential nutrients, such as N compounds, during the rapid fermentation of carbohydrates (El-Shazly *et al.* 1961).

Despite the widespread application of real-time PCR technology in quantitative PCR assays, this method is relatively expensive and requires a high level of technical support. Based on our results, this technique could be replaced, at least in many studies, with the simpler less technologically demanding QC-PCR. It provides a relatively quick simple and reliable method for the relative quantification of rumen anaerobic fungi populations and is suitable for use in laboratories with low budgets.

Acknowledgements

We thank Hossien Tavassoli, Amir Mohammadi, and Ali Javadmanesh for technical assistance with microbiology and molecular genetics. This work was financially supported by the Excellence Center for Animal Science, Ferdowsi University of Mashhad.

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