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# Anaerobic Cellulolytic Rumen Fungal Populations in Goats Fed with and without *Leucaena leucocephala* Hybrid, as Determined by Real-Time PCR

**Ching Mun Kok[1], Chin Chin Sieo[1,2], Hui Yin Tan[1], Wan Zuhainis Saad[2], Juan Boo Liang[3], and Yin Wan Ho[1]***

*[1]Institute of Bioscience, [2]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, [3]Institute of Tropical Agriculture, Universiti Putra Malaysia, Selangor, 43400, Malaysia*

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**The effect of *Leucaena leucocephala* hybrid-Bahru (LLB), which contains a high concentration of condensed tannins, on cellulolytic rumen fungal population in goats was investigated using real-time PCR. The fungal population in goats fed LLB was inhibited during the first 10 days of feeding, but after 15 days of feeding, there was a tremendous increase of fungal population (157.0 μg/ml), which was about four-fold more than that in control goats (39.7 μg/ml). However, after this period, the fungal population decreased continuously, and at 30 days of feeding, the fungal population (50.6 μg/ml) was not significantly different from that in control goats (55.4 μg/ml).**

***Keywords***: *Leucaena*, condensed tannins, rumen fungal population, real-time PCR

A major problem facing farmers in tropical region is poor livestock productivity due to poor quality feedstuffs. *Leucaena leucocephala*, a tropical legume that contains a high level of crude protein, is widely used as a protein supplement for ruminants. However, an outbreak of psyllid (*Heteropsylla cubana*) attack on *L. leucocephala* in the 1980s almost wiped out the plants in the region. In Malaysia, *L. leucocephala* hybrids that are psyllid-resistant are found to have higher concentrations of condensed tannins (CTs) and lower dry matter digestibility than the parent *L. leucocephala*. It has been suggested that the lower digestibility is due to the higher CT content in the hybrids (Liang and Khamseekhiew, 2006). However, it is not known whether cellulolytic rumen fungi, which play a major role in digestion of feed materials, are affected by the *L. leucocephala* hybrids.

*For correspondence. E-mail: yhwo@upm.edu.my; Tel. & Fax: +060-3-8947-2161

Anaerobic cellulolytic rumen fungi have been recognised as the primary colonisers of plant cell walls (Gordon and Phillips, 1993). The classical microbiological methods of enumerating rumen fungi are by zoospore count and by the most probable number method which estimates thallus forming units (Theodorou *et al.*, 1990; Hespell *et al.*, 1997). Both these methods may underestimate the fungal population, especially the polycentric species which could form new thalli from old rhizomycelial fragments instead of zoospores (Denman and McSweeney, 2006). A more recent molecular method using real-time PCR assay was developed by Denman and McSweeney (2006) to enumerate the population of anaerobic rumen fungi in order to overcome the underestimation of the classical microbiological methods. Earlier, Bowman *et al.* (1992) had reported that the 18S rRNA gene sequence is highly conserved within the fungi. This character helps in real-time PCR assay to generate amplicons with similar sequences which are able to produce a more defined dissociation peak. Several studies have shown that real-time PCR assay can be used on rumen fluid and digesta samples to monitor populations and diversity of rumen microbes (Sekhavati *et al.*, 2009; Yang *et al.*, 2010; Tan *et al.*, 2011). In the present study, the effect of *L. leucocephala* hybrid-Bahru (LLB), which is CT-rich, on the cellulolytic rumen fungal population in goats was investigated using real-time PCR.

Eight Boer cross Saanen male goats, 12 months old, with average body weight of 26.28±2.23 kg, were used as experimental animals. They were housed in individual pens (1.2 m × 2 m). Four goats were randomly assigned to one of two diets: (i) control diet without LLB and (ii) treatment diet with LLB. The compositions of the two diets (Table 1) were formulated according to NRC (1981) and chemical compositions were analysed based on the official methods of analysis from the Association of Official Analytical Chemists (AOAC, 1990), and Van Soest *et al.* (1991) to ensure that the nutritional contents of both diets were similar. Feed samples for chemical composition were dried at 55°C, then ground using a 1 mm screen and analysed for dry matter, crude protein, ash, ether extract (AOAC, 1990), neutral detergent fiber and acid detergent fiber (Van Soest *et al.*, 1991). Proximate analyses of the chemical composition of components of the control and treatment diets showed that there were no significant ($P$>0.05) differences in the nutritional contents of the two diets (Table 1). The goats were

**Table 1. Composition of diets**

| Item (DM) | Diet | |
|---|---|---|
| | Control | Treatment |
| Ingredient (%) | | |
| Commercial goat pellets | 40 | 40 |
| Alfalfa hay | 30 | 5 |
| Soybean hull pellet | 20 | 15 |
| Guinea grass (*Panicum maximum*) | 10 | 10 |
| *Leucaena leucocephala* hybrid-Bahru | - | 30 |
| Total | 100 | 100 |
| Concentration of condensed tannins from *Leucaena leucocephala* hybrid-Bahru in diet | 0 | 1.8 |
| Chemical composition (%) | | |
| Dry matter | 93.18 | 93.30 |
| Crude protein | 16.15 | 17.64 |
| Crude fibre | 18.63 | 18.59 |
| Acid-detergent fibre | 30.85 | 28.40 |
| Neutral-detergent fibre | 51.80 | 48.55 |
| Acid-detergent lignin | 19.57 | 25.95 |
| Ether extract | 0.94 | 0.96 |
| Ash | 6.67 | 6.22 |
| Gross energy | 16.74 MJ/kg DM | 17.15 MJ/kg DM |

given feed at 2.7% dry matter (DM) of mean body weight per day (Sidahmed *et al*., 1983). Young leaves and shoots of approximately 8-week re-growth of LLB were harvested between 09:00 to 10:30 h from the research farm of the Department of Animal Science, Universiti Putra Malaysia (3°00′18.88″ N 101°42′15.05″ E). Harvesting of re-growth at about 8 weeks was recommended to obtain the highest nutrient yield (Garcia *et al*., 1996). The CT content of the hybrid was found to be 6% (DM) CTs (Tan H.Y., unpublished data). The samples were air dried and the dried leaves and shoots were chopped to 5 cm lengths before mixing with the feed. Guinea grass (*Panicum maximum*) was cut every morning at 08:30 h from the same research farm. Alfalfa hay (Persahabatan Ltd. Co., Malaysia), soybean hull pellets (Soon Soon Oilmills Ltd. Co., Malaysia) and commercial goat pellets (Persahabatan Ltd. Co.) were purchased. The goats were fed twice daily, at 10:00 and 17:00 h, and had free access to water and mineral blocks. The experiment was carried out twice using a crossover design. Each experimental period was 30 days. Prior to the experiment, and between experimental periods, there were 2 weeks of adaptation during which the goats were fed a basal diet that was similar to the control diet. All animal management and sampling procedures were approved by the Universiti Putra Malaysia Animal Care and Use Committee.

Rumen fluid and digesta were taken from each goat at 5-day intervals for 30 days. Approximately 50 ml of rumen sample was taken from each goat via a stomach tube attached to a vacuum pump at 09:00 h (16 h after feeding), and placed in a warm (39°C) insulated flask pre-gassed with carbon dioxide. In the laboratory, the samples were separately strained through four layers of muslin. About 0.25 g of strained rumen sample was taken using a wide-bore pipette to ensure that a homogeneous sample containing fine plant particles and liquid was obtained. DNA was extracted using the QIAamp® DNA stool kit (QIAGEN®) according to the manufacturer's instructions with slight modifications. The procedure of extraction was modified by adding 0.4 g of 0.1 mm glass beads into the samples. This was to ensure that fungal cell walls were broken effectively, yielding maximum DNA. From the kit, 50 µl of pure total rumen microbial DNA was eluted. Eluted DNA (concentration of about 200 µg/ml) to be used as a template for real-time PCR assay was diluted 10 times to reduce inhibition (Denman and McSweeney, 2006).

The primers used for detecting the total rumen fungal population were the same as those designed by Denman and McSweeney (2006). The forward primer sequence was 5′-GAG GAA GTA AAA GTC GTA ACA AGG TTT C-3′ and the reverse primer sequence was 5′-CAA ATT CAC AAA GGG TAG GAT GAT T-3′, with an expected amplicon size of 120 bp. This primer set used for real-time PCR was designed from the 18S rRNA gene and ITS1 region, which included representatives of closely and distantly related fungal species. The primers were designed to target a region that is highly conserved. Real-time PCR assay validation for the primers had previously been carried out by Denman and McSweeney (2006). Positive results were observed for anaerobic cellulolytic rumen fungi but not other aerobic chytrid fungi. In the current study, validation of the primers was also carried out using conventional PCR. A single specific band of about 120 bp (expected size) was observed in the 2% agarose gel and there were no primer dimer products. This indicated the specificity of the primer pair used. Absolute quantification of anaerobic cellulolytic rumen fungi was carried out based on the standard curve method in real-time PCR. DNA from a pure cellulolytic rumen fungus, *Piromyces* sp. PGL25, was used for preparation of the standard curve. *Piromyces* sp. PGL25 was isolated from the rumen of a goat fed LLB in the present study, and was identified using morphological characteristics (Ho and Barr, 1995) and the 18S rRNA gene and ITS1 region sequences. The 18S rRNA gene and ITSI region sequences were deposited in the GenBank under the accession numbers HQ 585900 and HQ 585904, respectively. A standard curve was constructed by plotting the observed threshold cycle ($C_t$) values against the loga-

**Table 2. Results of analysis of variance (ANOVA) of rumen fungal biomass in goats fed with (treatment) or without (control) *L. leucocephala* hybrid-Bahru, estimated using real-time PCR**

| Source | df | Mean square | Probability |
|---|---|---|---|
| Diet | 1 | 1.701 | * |
| Day | 6 | 6340.03 | * |
| Diet x Day | 6 | 4885.46 | * |
| Error | 99 | 3.42 | - |
| Total | 112 | - | - |

* $P<0.05$

rithm of DNA concentration per ml (ng/ml) of *Piromyces* sp. PGL25 in the real-time PCR assay. The standard curve plotted had an efficiency of 95% (slope value: -3.45), with Y-intercept at 36.12 and $R^2$ value of 0.99. A good reaction should have an efficiency between 90% and 110%, which corresponds to a slope of between -3.6 and -3.1 and $R^2$ value should be ≥0.985. Therefore, the efficiency of 95% and the $R^2$ value of 0.99 for the standard curve in this study were within the acceptable range. A linear standard curve indicates that the efficiency of the reaction is constant throughout the range of template concentrations. A single dissociation peak was observed at 76°C. This indicated that the amplicons contained similar sequence alignments. Real-time PCR was performed using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, USA). The reaction mixture consisted of an optimal primer concentration of 300 nM, 4 µl of DNA template and Power SYBR® Green PCR Master Mix. Three replicates were made for each sample. Real-time PCR cycling conditions consisted of one cycle at 50°C for 2 min for activation of hotstart DNA polymerase, 95°C for 2 min for denaturation, 40 cycles at 95°C for 15 sec and 60°C for 1 min for primer annealing and product elongation. At the end of each denaturation and extension step, fluorescence detection was performed. Amplicon specificity was performed by increasing the temperature at the rate of 1°C every 30 sec from 60 to 95°C to obtain the dissociation curve. Real-time PCR was carried out four times to ensure accuracy of results. To guard against inaccurate results due to contaminations, at least three no-template control (NTC) wells were employed. In these wells, DNA template was replaced by sterile distilled water. Quantification was carried out by comparing the $C_t$ of the DNA template with that of the standard.

Statistical analysis of results was performed using SPSS 16.0 software (SPSS Inc., 2002). Significant differences in the different diets, days of the experimental period and their interactions (diet × day) were tested using two-way analysis of variance (ANOVA) with the initial record measured at day 0 as covariate. Differences in the chemical compositions of the diets were analysed using the *t*-test. Significant differences between means were analysed using *post hoc* tests at $P<0.05$.

Real-time PCR has been used to quantify fungal DNA in environmental samples such as soil, plant and wine (Filion *et al*., 2002; Lievens *et al*., 2006; Selma *et al*., 2008). However, conversion of fungal DNA to fungal biomass in these environmental samples has not been carried out. Denman and McSweeney (2006) in their study in enumerating rumen bacteria and fungi using real-time PCR assay were the first to show that 13 µg of fungal DNA was equivalent to 1 mg of fungal biomass. In the present study, data from real-time PCR quantification of fungal populations were expressed as fungal biomass (µg/ml) following the method of Denman and McSweeney (2006). Analysis of the results showed that there were significant ($P<0.05$) effects of days of experiment, diets, and their interaction (day × diet) on the fungal population (Table 2). Figure 1 shows the anaerobic cellulolytic rumen fungal population patterns in goats fed with or without LLB during the 30-day experimental period. In goats fed control diet, there were some fluctuations in the fungal population during the experimental period. The fungal population increased ($P<0.05$) from 11.2 µg/ml at day 0 to 41.8 µg/ml at day 20, then decreased to 28.4 µg/ml at day 25, after which there was an increase ($P<0.05$) again to 55.4 µg/ml at day 30. For treatment goats, which were fed CT-rich LLB, the fungal population decreased significantly ($P<0.05$) from 17.9 µg/ml at day 0 to 12.4 µg/ml at day 5 and 8.6 µg/ml at day 10. However, after this period, the fungal population increased tremendously ($P<0.05$) to 157.0 µg/ml at day 15. This was nearly four-fold more than the fungal population in control goats (39.7 µg/ml) during the same period. After this, the fungal population decreased ($P<0.05$) again to 124.5, 80.0, and 50.6 µg/ml at days 20, 25, and 30, respectively. At day 30, the fungal biomass in treatment goats (50.6 µg/ml) was not significantly different from that in control goats. The

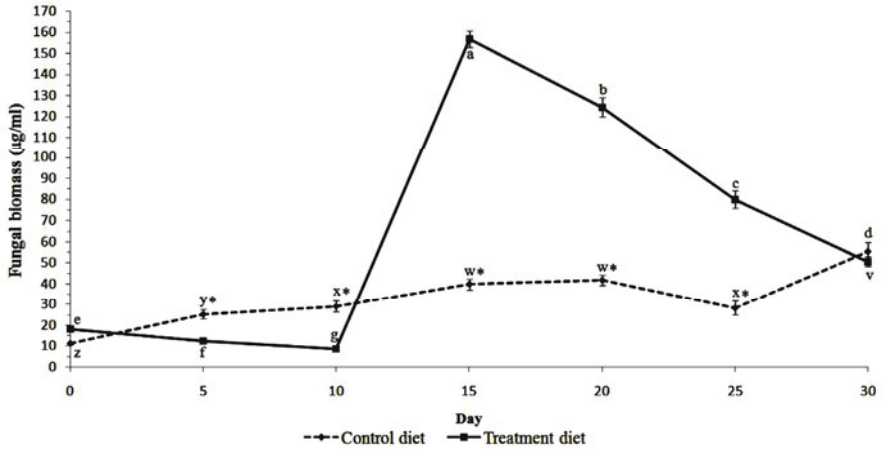


**Fig. 1. Biomass of rumen fungi in goats fed with (treatment) or without (control) *L. leucocephala* hybrid-Bahru, estimated using real-time PCR.** Bar represents Means±SD of 8 goats in each diet. [a-f] Means in treatment diet with different letters are significantly different ($P<0.05$). [v-z] Means in control diet with different letters are significantly different ($P<0.05$). *Means between diets on the same day are significantly different ($P<0.05$).

results indicated that the growth of cellulolytic rumen fungi might have been inhibited by the CTs of LLB during the initial 10 days of feeding, but after this period, the rumen fungi could have adapted to the CTs and growth seemed to be stimulated, resulting in a tremendous increase of fungal biomass at 15 days of feeding. However, the rapid growth was not sustained with prolonged exposure to CTs of LLB. So far, there has been no study on the effect of *Leucaena* CTs on the anaerobic rumen fungal growth pattern during different periods of supplementation for us to compare. Nevertheless, there have been some studies on the effect of tannins from other plant sources on rumen microbial populations. McSweeney *et al*. (2001a) reported that 30% of Calliandra (which contains 6% CTs) in the diet of sheep reduced the population of cellulolytic rumen bacteria (*Fibrobacter* and *Ruminococcus*) by approximately 6%, but the effect of Calliandra CTs on cellulolytic rumen fungi was less clear as the results were inconsistent. Earlier, Chiquette *et al*. (1988) found that dietary tannins might prevent colonisation of leaf materials by rumen microbes. McSweeney *et al*. (2001b) also suggested that microbial populations in the rumen were not only dependent on the availability of nutrients but also on their tolerance to tannins in the feed. However, the mechanism of how rumen microbes adapt to tannins is still unclear. It has been reported that extracellular polysaccharides which have high affinity for binding to tannins are secreted by rumen microbes and these polysaccharides, which can form a thick high-tannin-affinity glycoprotein, could prevent tannins causing adverse effects on the microbes (Chiquette *et al*., 1988; Brooker *et al*., 2000).

In conclusion, the results of the present study showed that LLB containing 6% CTs fed to goats affected the population of cellulolytic rumen fungi at different periods of feeding, with inhibition of growth at the early period, followed by adaptation and proliferation of growth, then reduction again with prolonged exposure to CTs.

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