



UNIVERSITI PUTRA MALAYSIA

***EFFECTS OF CONDENSED TANNINS FROM LEUCAENA
LEUCOCEPHALA (LAM.) DE WIT HYBRID ON POPULATION AND
CELLULOLYTIC ACTIVITY OF RUMEN FUNGI***

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**EFFECTS OF CONDENSED TANNINS FROM *Leucaena leucocephala* HYBRID
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RUMEN FUNGI**



By

KOK CHING MUN

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Condensed tannins (CT), which are secondary metabolites of *Leucaena leucocephala*, have both beneficial and harmful effects on ruminants. CT levels that do not exceed 5% dry matter (DM) could prevent bloat as well as provide bypass-protein to the animal. However, high levels of CT could reduce the nutritional value of *Leucaena*, decrease N digestibility and depress rumen digestion of fermentable carbohydrates. *L. leucocephala* hybrids, which are resistant to psyllid infestation, have been found to contain high CT levels and exhibited low digestibility in ruminant. It has been suggested that the low digestibility is attributed to the high CT content. However, it is not known whether the cellulolytic anaerobic rumen fungi, which play a major role in the digestion of fibrous feed materials, are affected by the CT from *L. leucocephala* hybrids. Thus, the objectives of this study were to determine the effects of a *L. leucocephala* hybrid (Bahru) containing high CT level on the population and cellulolytic activity of rumen fungi. The

animals used in the study were eight Boer cross Saanen male goats. Four goats were randomly assigned to one of two diets: (i) control diet without *L. leucocephala* hybrid-Bahru and (ii) treatment diet containing 30% *L. leucocephala* hybrid-Bahru, which was equivalent to 1.8% CT of dietary DM. The goats were fed with 2.7% DM of mean body weight per day. The experiment was carried out twice using a cross over design and each experimental period was 30 days. Total rumen fungal population (expressed as fungal biomass) was estimated using real-time PCR. The results showed that the cellulolytic rumen fungal population of goats fed with *L. leucocephala* hybrid-Bahru decreased significantly at days 5 and 10 (12.38 µg/ml and 8.63 µg/ml), and then increased drastically at day 15 (157.00 µg/ml). However, the rapid growth was not sustained and the rumen fungal population decreased again at days 20 and 25 of feeding, and at day 30, the fungal population of treatment goats was not significantly different from that of the control goats. These results showed that growth of the cellulolytic rumen fungi was inhibited by the CT of *L. leucocephala* hybrid-Bahru during the initial period of feeding, but after this period, the fungi could have adapted to the CT and thrived, resulting in an enormous increase in fungal growth at day 15. However, the rapid growth was not sustained with prolonged exposure to CT of *L. leucocephala* hybrid-Bahru. Further studies using pure CT extracted from *L. leucocephala* hybrid-Bahru were then carried out to determine the effects of pure CT on the enzyme activities of some isolated cellulolytic rumen fungi. One hundred and twenty rumen fungi were isolated and identified to the genus level based on gross morphological characteristics. All the rumen fungal isolates identified belonged to two genera only, namely, *Neocallimastix* and *Piromyces*. Four highly cellulolytic fungal isolates were then selected based on their rate

of degradation of ball-milled filter paper (one *Neocallimastix* and one *Piromyces* isolated from treatment goats at day 0, and one *Neocallimastix* and one *Piromyces* isolated from treatment goats at day 25). The four selected highly cellulolytic *Neocallimastix* and *Piromyces* isolates were further identified to species level using detailed morphological and molecular characteristics, such as sequence analysis of the 18S rRNA gene and ITS1 region. The two *Neocallimastix* isolates were identified as *Neocallimastix frontalis* (NGL 01 from day 0 and NGL 25 from day 25 of feeding, respectively) but the two *Piromyces* isolates could only be identified as *Piromyces* sp. (PGL 01 from day 0 and PGL 25 from day 25 of feeding, respectively). Enzyme assays were carried out to estimate the effects of pure CT on the cellulolytic enzyme activities and fermentation end products of the four isolated fungi. Four enzyme activities were studied. They were carboxymethylcellulase (CMCase), filter paperase (FPase), xylanase and β -glucosidase. The results showed that enzyme activities of cellulolytic rumen fungi isolated from goats fed *L. leucocephala* hybrid-Bahru at day 25 (*N. frontalis* NGL 25 and *Piromyces* sp. PGL 25), and had been exposed to CT in the rumen, were numerically or significantly more than those of cellulolytic rumen fungi isolated from goats not fed with *L. leucocephala* hybrid-Bahru (*N. frontalis* NGL 01 and *Piromyces* sp. PGL 01) at all levels of CT inclusion, which indicate greater tolerance of CT in cellulolytic rumen fungi isolated from goat fed *L. leucocephala* hybrid-Bahru at day 25 (*N. frontalis* NGL 25 and *Piromyces* sp. PGL 25). In conclusion, CT from *L. leucocephala* hybrid-Bahru could affect population and cellulolytic enzyme activities of anaerobic rumen fungi.

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KESAN TANIN TERKONDENSASI DARI *Leucaena leucocephala* HIBRID TERHADAP POPULASI DAN AKTIVITI SELULOLITIK OLEH KULAT RUMEN

Oleh

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April 2012

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Tanin terkondensasi (CT), adalah metabolit sekunder dari *Leucaena leucocephala*, yang mempunyai kedua-dua kesan bahaya dan juga bermanfaat pada ruminan. Tahap CT yang tidak melebihi 5% jirim kering boleh menghalang pengembangan serta membekalkan protein pintasan bagi haiwan. Walau bagaimanapun, tahap CT yang tinggi boleh mengurangkan zat makanan *Leucaena*, keupayaan penghadaman nitrogen dan merencat penghadaman fermentasi karbohidrat pada rumen. *L. leucocephala* hibrid, yang rintang terhadap serangan psyllid, didapati mengandungi tahap CT yang tinggi dan menunjukkan penghadaman yang rendah pada ruminan. Didapati bahawa tahap penghadaman yang rendah dianggap berpunca daripada kandungan CT yang tinggi. Bagaimanapun, ia tidak diketahui sama ada selulolitik kulat rumen, yang memainkan peranan utama dalam pencernaan bahan makanan berserabut, akan terjejas oleh CT daripada hibrid *L. leucocephala*. Oleh itu, objektif kajian ini untuk menentukan kesan hibrid *L. leucocephala* (Bahru) yang mangandungi tahap CT yang tinggi, terhadap populasi dan aktiviti

selulolitik kulat rumen. Haiwan yang digunakan dalam kajian ini adalah lapan kambing jantan kacukan Boer dan Saanen. Empat kambing dibahagikan secara rawak kepada satu daripada dua diet: (i) diet kawalan tanpa *L. leucocephala* hibrid-Bahru dan (ii) diet rawatan mengandungi 30% *L. leucocephala* hibrid-Bahru, atau persamaan dengan CT 1.8% CT DM pemakanan. Kambing diberi makanan mengandungi 2.7% DM daripada min berat badan setiap hari. Eksperimen dijalankan dua kali menggunakan reka bentuk pindah silang dan tempoh setiap eksperimen adalah 30 hari. Jumlah populasi kulat rumen (dinyatakan sebagai biojisim kulat) dianggarkan menggunakan 'real-time PCR'. Keputusan menunjukkan populasi selulolitik kulat rumen pada kambing yang diberi makanan *L. leucocephala* hibrid-Bahru menurun dengan signifikan pada hari 5 dan 10 (12.38 µg/ml and 8.63 µg/ml), dan kemudian meningkat secara drastik pada hari 15 (157.00 µg/ml). Walaubagaimanapun, pertumbuhan pesat tidak berkekalan dan populasi kulat rumen menurun semula pada hari 20 dan 25 pemakanan, dan pada hari 30, populasi kulat rumen dari kambing rawatan tidak berbeza secara signifikannya daripada populasi kambing kawalan. Keputusan ini menunjukkan bahawa pertumbuhan selulolitik kulat rumen telah dihalang oleh CT daripada *L. leucocephala* hibrid-Bahru semasa tempoh permulaan pemakanan, tetapi selepas tempoh itu, kulat mungkin telah menyesuaikan diri dengan CT dan bertumbuh dengan pesat, mengakibatkan peningkatan yang besar dalam pertumbuhan kulat pada hari 15. Walau bagaimanapun, pertumbuhan pesat berkekalan dengan pendedahan yang berlanjutan kepada CT daripada *L. leucocephala* hibrid-Bahru. Lanjutan kajian menggunakan CT tulen yang diekstrak daripada *L. leucocephala* hibrid-Bahru kemudian dijalankan untuk menentukan kesan CT tulen pada aktiviti enzim daripada beberapa selulolitik kulat rumen yang terpencil. 120 kulat rumen yang terpencil, dikenal pasti kepada tahap genus berdasarkan ciri-ciri morfologi kasar. Kesemua kulat rumen yang terpencil dikenal pasti berasal daripada dua genera, iaitu *Neocallimastix* and

Piromyces. Empat berkemungkinan selulolitik kulat rumen akan dipilih berdasarkan kadar degradasi pada media kertas turas (satu *Neocallimastix* dan satu *Piromyces* yang dipencil daripada kambing rawatan pada hari 0, dan satu *Neocallimastix* dan satu *Piromyces* yang dipencil daripada kambing rawatan pada hari 25). Empat berkemungkinan selulolitik kulat rumen *Neocallimastix* dan *Piromyces* yang terpencil akan dikenal pasti secara terperinci kepada peringkat spesies menggunakan ciri-ciri morfologi dan molekul, seperti analisis urutan gen 18S rRNA dan rantau ITS1. Kedua-dua *Neocallimastix* yang terpencil dikenal pasti sebagai *Neocallimastix frontalis* (NGL 01 daripada hari 0 dan NGL 25 daripada hari 25) tetapi, dua *Piromyces* yang terpencil hanya boleh dikenal pasti sebagai *Piromyces* sp. (PGL 01 daripada hari 0 dan PGL 25 daripada hari 25). Ujian enzim telah dijalankan untuk menganggar kesan CT tulen pada aktiviti enzim selulolitik dan produk akhir fermentasi kulat rumen terpencil. Empat enzim akan dikaji. Ia adalah karbosimetiselulase (CMCase), filterpaperase (FPase), xilanase dan β -glukosidase. Hasil kajian menunjukkan aktiviti enzim pada selulolitik kulat rumen yang terpencil pada hari 25 (*N. frontalis* NGL 25 dan *Piromyces* sp. PGL 25), yang telah terdedah kepada CT dalam rumen, adalah lebih tinggi secara signifikannya daripada selulolitik kulat rumen yang terpencil daripada kambing yang tidak diberi pemakanan dengan *L. leucocephala* hibrid-Bahru (*N. frontalis* NGL 01 dan *Piromyces* sp. PGL 01) pada semua peringkat CT, di mana menunjukkan rintangan yang lebih tinggi kepada CT pada selulolitik kulat rumen yang telah dipencil daripada kambing yang diberi pemakanan *L. leucocephala* hibrid-Bahru pada hari 25 (*N. frontalis* NGL 25 dan *Piromyces* sp. PGL 25). Kesimpulannya, CT daripada *L. leucocephala* hibrid-Bahru boleh mempengaruhi populasi dan aktiviti selulolitik enzim pada aktiviti anaerobik kulat rumen.

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It's fine to celebrate success but it is more important to heed the lessons of failure.

- Bill Gates, Chairman of Microsoft.

I certify that an Examination Committee has been on **3 April 2012** to conduct to conduct the final examination of **Kok Ching Mun** on her **Master of Science** thesis entitled “**Effects of Condensed Tannins from *Leucaena leucocephala* (Lam.) de Wit Hybrid on Population and Cellulolytic Activity of Rumen Fungi**” in accordance with Universities and Unbiversity Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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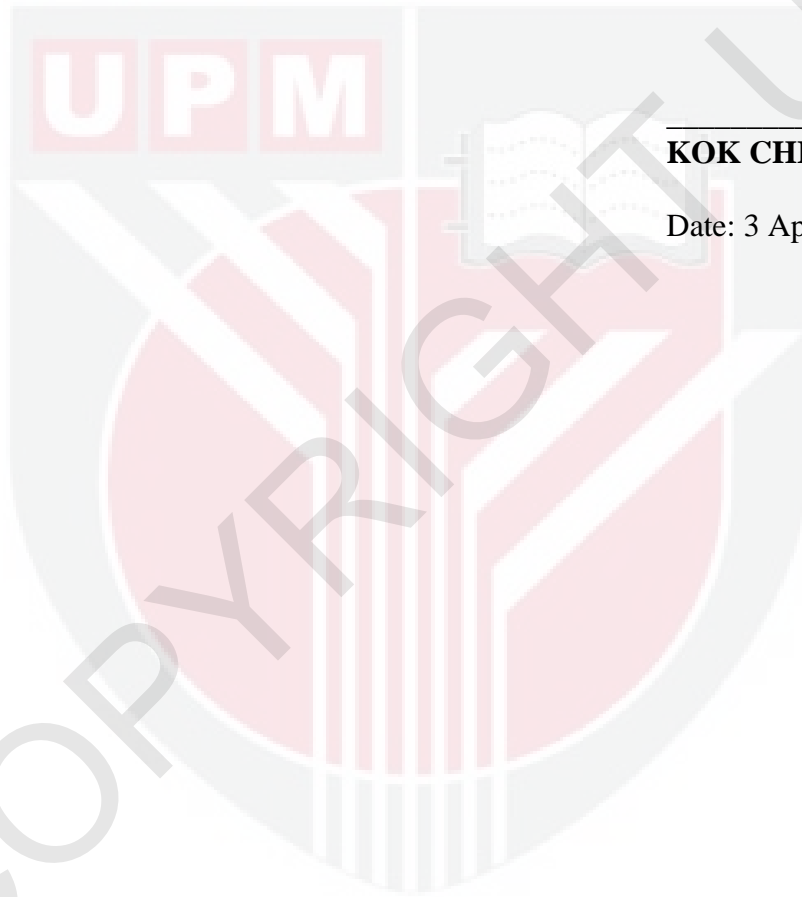
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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institutions.



KOK CHING MUN

Date: 3 April 2012

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LIST OF ABBREVIATIONS

CT	Condensed tannins
VFA	Volatile fatty acids
Non-VFA	Non-volatile fatty acids
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
PCR	Polymerase chain reaction
ITS1	Internal transcribed space 1
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
FPase	Filter paperase
BW	Body weight
DM	Dried matter
CP	Crude protein
CF	Crude fiber
ADF	Acid detergent fiber
NDF	Neutral detergent fiber
ADL	Acid detergent lignin
EE	Ether extract
GE	Gross energy
β	Beta

CHAPTER 1

INTRODUCTION

A major problem facing farmers in tropical regions, including Malaysia, is poor livestock productivity due to poor quality feedstuffs. Low nitrogen and high fiber contents of tropical grasses and crop residues are insufficient to promote growth and productivity of livestock. Feeding of legume forages, which are usually characterised by high crude protein and mineral contents, has been practised as a cheaper option in order to increase nitrogen intake and digestibility (Fondevila *et al.*, 2002). The legume forages can be degraded rapidly in the rumen, supplying fermentable nitrogen and carbohydrates to the host animal.

Leucaena leucocephala, a tropical legume which contains high amount of crude protein, is widely used as a protein supplement for ruminants in tropical regions. However, an outbreak of psyllid insects (*Heteropsylla cubana*) in the 1980s almost wiped out the plants in the region. In Malaysia, a psyllid resistant *L. leucocephala* hybrid was developed by crossing *L. leucocephala* and *L. diversifolia* (Wong *et al.*, 1998). However, high levels of tannins and low digestibility were detected in the hybrids fed to ruminant (Khamseekhiew, 2006). Tannins are classified into two groups, hydrolysable tannins and condensed tannins, based on their chemical and structural properties. Hydrolysable tannins can be easily hydrolysed by enzymes or acids into simple monomers. Condensed tannins are not susceptible to enzyme degradation (McSweeney *et al.*, 2001a) and have the ability to bind with proteins to form tannin-protein complexes. Condensed tannins have both beneficial and harmful effects on ruminants. Levels of condensed tannins

which are lower than 5% dry matter are beneficial to the animal in preventing bloat and providing valuable rumen bypass-protein by reducing rumen degradation of protein in the form of condensed tannin-protein complexes which are resistant to rumen microbial degradation. However, high levels of condensed tannins (10-15% dry matter) can reduce the nutritional value of *Leucaena*, decrease nitrogen digestibility and depress rumen digestion of fermentable carbohydrates (Khamsekhiew, 2006).

The rumen consists of complex microbial populations comprising predominantly bacteria, fungi and protozoa which are involved in the digestion of feed materials. Among the rumen microbes, anaerobic rumen fungi are considered as the primary colonisers of fibrous plant materials (Gordon and Phillips, 1993). Anaerobic rumen fungi have the ability to utilise major plant cell wall polysaccharides for growth. They produce a wide range of cellulolytic enzymes to hydrolyse the components of plant cell walls. They are able to metabolise the plant cell wall and penetrate the cuticle more efficiently than rumen bacteria (Joblin *et al.*, 1989). Although Khamsekhiew (2006) suggested that the lower digestibility of *L. leucocephala* hybrids was probably due to their higher levels of condensed tannins, it is not known whether condensed tannins from *L. leucocephala* hybrids have adverse effects on cellulolytic rumen microbial populations, particularly the rumen fungi and their cellulolytic activities.

Thus, this study was conducted to investigate the effects of a *L. leucocephala* hybrid (Bahru) containing high condensed tannin level on the population of anaerobic rumen fungi *in vivo* and their cellulolytic enzyme activities *in vitro*.

The specific objectives were:

- (1) to estimate the population of anaerobic rumen fungi using molecular technique, such as real-time PCR assay, in goats fed with or without *L. leucocephala* hybrid-Bahru,
- (2) to isolate and identify anaerobic rumen fungi based on morphological and molecular characteristics, such as sequence analysis of the 18S rRNA gene and ITS1 region, and
- (3) to study the effects of pure condensed tannins extracted from *L. leucocephala* hybrid-Bahru on cellulolytic enzyme activities and fermentation end products of some anaerobic rumen fungi selected from (2) above.

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3. Kok, C.M., Sieo, C.C., Zuhainis, S.W., Liang, J.B. and Ho, Y.W. 2009. Identification of an anaerobic rumen fungus using 18S rRNA gene and ribosomal ITS1 region. Proceedings of Second International Conference of Sustainable Animal Agriculture for Developing Countries, 8-11 November 2009, Kuala Lumpur, pp 255-256.
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