UNIVERSITI PUTRA MALAYSIA

EFFECTS OF CONDENSED TANNINS FROM *LEUCAENA LEUCOCEPHALA* (LAM.) DE WIT HYBRID ON METHANE MITIGATION, RUMEN FERMENTATION, AND POPULATIONS OF METHANOGENS AND PROTOZOA *IN VITRO*

TAN HUI YIN

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By

TAN HUI YIN

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

March 2012

DEDICATION

This thesis is dedicated to my parents
for their love, endless support
and encouragement

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

EFFECTS OF CONDENSED TANNINS FROM *LEUCAENA LEUCOCEPHALA* (LAM.) DE WIT HYBRID ON METHANE MITIGATION, RUMEN FERMENTATION, AND POPULATIONS OF METHANOGENS AND PROTOZOA *IN VITRO*

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Chairman: Professor Ho Yin Wan, PhD

Institute : Bioscience

Methane is the second most important greenhouse gas that contributes to global warming and climate change. Methane production from livestock, predominantly ruminants, accounts to about one-third of global anthropogenic methane production. Methane produced during ruminal fermentation also represents a loss of gross energy consumed up to 12%. Thus, there is an urgent need to reduce methane emission from ruminants. This study was carried out to investigate the effects of condensed tannins (CTs), a plant secondary metabolite, from *Leucaena leucocephala* hybrid-Rendang (LLR) on methane mitigation, rumen fermentation, and molecular diversities and populations of methanogens and protozoa *in vitro*. The study was divided into four experiments.

In the first experiment, extraction, purification and determination of molecular weights of crude and pure CT extracts from LLR were carried out. It was found that

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the number-average molecular weights (M_n) determined using quadrupole time-of-flight mass spectrometer were 1087.99 and 1070.51 Da for crude and pure CT extracts, respectively.

In the second experiment, different levels of crude and pure CTs extracted from LLR were investigated for their effects on methane production and rumen fermentation parameters such as pH, dry matter degradability, nitrogen disappearance and volatile fatty acid concentrations. Crude CT concentrations of 0 (control), 10, 25, 40 and 55 mg, and 500 mg of oven dried guinea grass (*Panicum maximum*) with 40 ml of buffered rumen fluid were incubated for 24 h using an *in vitro* gas production procedure. Results showed that total gas production decreased linearly ($P<0.05$) with increasing inclusions of crude CT levels. A linear decrease ($P<0.01$) in methane production was observed at higher concentrations of crude CTs of 40 and 55 mg/500 mg DM, with reduction of 32.4% for both levels when compared to the control. Total volatile fatty acid concentration (mmol/L) decreased linearly ($P<0.01$) and propionate production increased at a linear rate ($P<0.01$) with additions of crude CTs. However, at 40 and 55 mg of crude CTs, *in vitro* dry matter (DM) degradation was significantly ($P<0.05$) reduced. Pure CT concentrations of 0 (control), 10, 15, 20, 25 and 30 mg were also studied using the *in vitro* gas production procedure. Results showed that total gas (ml/g DM) decreased (linear $P<0.01$; quadratic $P<0.05$) with increased levels of pure CT inclusion. Methane production (ml/g DM) decreased (linear $P<0.01$; quadratic $P<0.01$) with increasing levels of pure CTs. Total volatile fatty acid concentration (mmol/L) decreased linearly ($P<0.01$) and quadratically ($P<0.01$) with increasing pure CT inclusions. *In vitro* DM degradation and nitrogen disappearance declined linearly ($P<0.01$) with increasing levels of pure CTs. The

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results of the study showed that pure CTs from LLR at a relatively low level of 20 mg could reduce methane production by 57% without negatively affecting *in vitro* DM degradability and *in vitro* nitrogen disappearance. Although crude CTs and pure CTs at higher concentrations could also reduce methane emissions, they have substantive negative effects on DM digestibility.

Experiments three and four were carried out to estimate the populations and molecular diversities of methanogenic archaea and protozoa. Estimation of rumen methanogens and protozoal populations using microbiological methods and real-time PCR assay showed linear reductions in total methanogens ($P<0.01$) and total protozoa ($P<0.01$) with increasing levels of CTs. CT inclusion exhibited linear, quadratic and cubic effects on methanogens in the order *Methanobacteriales*.

Molecular diversities of rumen methanogens and protozoa from bovine rumen fluid incubated with pure CTs at 20 mg/500 mg DM or without CTs (control) was investigated using 16S rRNA and 18S rRNA gene libraries, respectively. The predominant order of rumen methanogens in the 16S rRNA gene libraries of the control and CT treatment was found to belong to a novel group of rumen archaea that is distantly related to the order *Thermoplasmatales*, with 59.5% (15 phylotypes) and 81.4% (21 phylotypes) of the total clones from the control and treatment clone libraries, respectively. The 16S rRNA gene library of the control was found to have higher proportions of methanogens in the orders *Methanomicrobiales* (32%) and *Methanobacteriales* (8.5%) as compared to those of the CT treatment clone library (16.9% and 1.7% respectively). The phylotype placed in the order

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Methanosarcinales was only found in the control clone library. The study indicated that CTs could alter the diversity of bovine rumen methanogens.

The 18S rRNA gene libraries of the protozoa revealed that all the clones were distributed in the orders *Entodiniomorphida* and *Trichostomatida*. A higher percentage of clones in the genus *Entodinium* (11.1% increase), and a lower percentage of clones in the genera *Ostracodinium* and *Anoplodinium* (5% and 3% decrease, respectively) were found in the CT treatment clone library.

In conclusion, the results indicated that pure CTs at the concentration of 20 mg/500 mg DM has the potential to be used as a feed supplement to reduce methane production by decreasing and altering the total methanogenic archaea and protozoal populations in ruminants, without adversely affecting DM degradability and nitrogen disappearance. However, *in vivo* experiments should be carried out to further evaluate the efficacy of CTs in methane mitigation.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KESAN-KESAN TANIN TERPELUWAP DARI *LEUCAENA LEUCOCEPHALA* (LAM.) DE WIT HIBRID TERHADAP MITIGASI METANA, FERMENTASI RUMEN SERTA POPULASI METHANOGEN DAN PROTOZOA *IN VITRO*

Oleh

TAN HUI YIN

Mac 2012

Pengerusi: Profesor Ho Yin Wan, PhD

Institut : Biosains

Metana ialah gas rumah hijau kedua terpenting yang menyumbang kepada pemanasan global dan perubahan iklim. Pengeluaran metana dari ternakan, khasnya ruminan, menyumbang kira-kira satu pertiga daripada pengeluaran metana antropogen global. Metana yang dihasilkan semasa fermentasi ruminan juga mewakili kerugian tenaga kasar daripada makanan sehingga 12%. Maka, adalah penting untuk mengurangkan penghasilan metana daripada ruminan. Kajian ini dijalankan untuk menyiasat kesan-kesan tannin terkondensasi (CT), sejenis metabolit sekunder tumbuhan, dari *Leucaena leucocephala* hybrid-Rendang (LLR) dalam pengurangan metana, fermentasi rumen, dan diversiti molekul serta populasi methanogen dan protozoa secara *in vitro*. Kajian ini dibahagikan kepada empat eksperimen.

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Dalam eksperimen pertama, pengekstrakan, penulenan dan penentuan berat-berat molekul ekstrak CT yang mentah dan tulen dari LLR telah dijalankan. Ia didapati nombor-purata berat-berat molekul sederhana (M_n) yang ditentukan menggunakan spektrometer jisim "quadruple time-of-flight" ialah 1087.99 dan 1070.51 Da, masing-masing untuk ekstrak CT yang mentah dan tulen.

Dalam eksperimen kedua, pelbagai tahap CT yang mentah dan tulen dari LLR telah dikaji untuk kesan-kesan pengeluaran metana serta parameter fermentasi rumen seperti pH, degradasi bahan kering, kehilangan nitrogen dan kepekatan asid lemak meruap. Kepekatan CT mentah iaitu 0 (kawalan), 10, 25, 40 dan 55 mg, dan 500 mg rumput kuda (*Panicum maksimum*) yang dikeringkan dengan ketuhar serta 40 ml bendalir rumen diinkubasi untuk 24 h menggunakan prosedur pengeluaran gas *in vitro*. Keputusan menunjukkan jumlah pengeluaran gas berkurang secara linear ($P<0.05$) dengan pertambahan tahap-tahap CT mentah. Pengurangan linear ($P<0.01$) dalam penghasilan metana telah diperhatikan di kepekatan-kepekatan CT mentah yang lebih tinggi, iaitu 40 dan 55 mg/500 mg DM, dengan pengurangan 32.4% untuk kedua-dua tahap berbanding dengan kawalan. Kepekatan jumlah asid lemak meruap (mmol/L) turun secara linear ($P<0.01$) dan penghasilan propionik bertambah pada suatu kadar linear ($P<0.01$) dengan penambahan CT mentah. Bagaimanapun, pada 40 dan 55 mg CT mentah, degradasi bahan kering *in vitro* (DM) nyata sekali ($P<0.05$) berkurang. Kepekatan CT tulen 0 (kawalan), 10, 15, 20, 25 dan 30 mg juga dikaji dengan menggunakan prosedur pengeluaran gas *in vitro*. Keputusan menunjukkan jumlah gas (ml/g DM) turun (linear $P<0.01$; kuadratik $P<0.05$) dengan kenaikkan tahap penambahan CT tulen. Penghasilan metana (ml/g DM) turun (linear $P<0.01$; kuadratik $P<0.01$) dengan peningkatan tahap-tahap CT

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tulen. Kepekatan jumlah asid lemak meruap (mmol/L) turun secara linear (P<0.01) dan kuadratik (P<0.01) dengan pertambahan CT tulen. Degradasi DM *in vitro* dan kehilangan nitrogen turun secara linear (P<0.01) dengan peningkatan tahap-tahap CT tulen. Keputusan-keputusan kajian menunjukkan CT tulen dari LLR pada satu tahap yang agak rendah iaitu 20 mg dapat mengurangkan penghasilan metana sebanyak 57% tanpa kesan pengaruh negatif ke atas degradasi DM *in vitro* dan kehilangan nitrogen *in vitro*. Walaupun CT mentah dan CT tulen pada kepekatan-kepekatan yang lebih tinggi boleh mengurangkan penghasilan metana, namun kesan negatif yang substantif pada degradasi DM telah ditunjukkan.

Eksperimen tiga dan empat dijalankan untuk menganggar populasi dan diversiti molekul archaea methanogenik dan protozoa. Anggaran methanogen rumen dan populasi protozoal menggunakan kaedah-kaedah mikrobiologi dan asai PCR masa nyata menunjukkan pengurangan linear dalam jumlah methanogen (P<0.01) dan jumlah protozoa (P<0.01) dengan peningkatan tahap-tahap CT. Penambahan CT menunjukkan kesan-kesan linear, kuadratik dan kubik pada methanogen dalam order *Methanobacteriales*.

Diversiti molekul methanogen rumen dan protozoa dari bendalir rumen yang diinkubasi dengan CT tulen 20 mg/500 mg DM atau tanpa CT (kawalan) dikaji dengan menggunakan gen perpustakaan 16S rRNA and 18S rRNA, masing-masing. Order utama methanogen rumen dalam gen perpustakaan 16S rRNA kawalan dan rawatan CT didapati berkait dengan sekumpulan archaea rumen novel yang berkaitan dengan methanogen dari order *Thermoplasmatales*, dengan 59.5% (15 phylotypes) dan 81.4% (21 phylotypes) daripada jumlah klon dari klon perpustakaan

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kawalan dan rawatan, masing-masing. Gen perpustakaan 16S rRNA kawalan didapati mempunyai pecahan methanogen yang lebih tinggi dari order *Methanomicrobiales* (32%) dan *Methanobacteriales* (8.5%) apabila dibanding dengan klon perpustakaan rawatan CT (16.9% dan 1.7% masing-masing). Phylotype yang berkait dengan order *Methanosarcinales* hanya ditemui dalam klon perpustakaan kawalan. Kajian menunjukkan CT boleh mengubah diversiti methanogen pada rumen.

Gen perpustakaan 18S rRNA protozoa menunjukkan bahawa semua klon tergolong dalam order *Entodiniomorphida* dan *Trichostomatida*. Peratusan yang lebih tinggi untuk klon-klon dari genus *Entodinium* (peningkatan 11.1%), dan pengurangan peratusan klon-klon dalam genera *Ostracodinium* and *Anoplodinium* (5% dan 3% pengurangan, masing-masing) telah ditemui dalam klon perpustakaan rawatan CT.

Kesimpulannya, keputusan-keputusan menunjukkan CT tulen pada kepekatan 20 mg/500 mg DM mempunyai potensi untuk digunakan sebagai satu makanan tambahan untuk mengurangkan penghasilan metana dengan mengurangkan dan meminda jumlah dan populasi archaea methanogenik dan protozoal pada ruminan, tanpa menjejaskan degradasi DM dan kehilangan nitrogen. Bagaimanapun, eksperimen-eksperimen *in vivo* sepatutnya dijalankan untuk seterusnya menilai kemujaraban CT dalam mitigasi metana.

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Thank you all for everything. Do enjoy life and may all your dreams come true.

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I certify that an Examination Committee met on **date of viva voce** to conduct the final examination of **Tan Hui Yin** on her **Doctor of Philosophy** thesis entitled "Effects of Condensed Tannins from *Leucaena leucocephala* Hybrid on Methane Mitigation, Rumen Fermentation and Populations of Methanogens and Protozoa *In Vitro*" in accordance with the Universities and University College Acte 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 Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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 other institutions.

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TABLE OF CONTENTS

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