EVALUATION OF POLYPHENOL RICH PAPAYA (CARICA PAPAYA L.) LEAF AND ITS EXTRACTS ON RUMEN MICROBES, BIOHYDROGENATION AND METHANE PRODUCTION

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By

SAEID JAFARI

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

March 2016

DEDICATION

I dedicate this work to my family. This

dissertation exists because of their love, support and prayers.

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

EVALUATION OF POLYPHENOL RICH PAPAYA (*CARICA PAPAYA* L.) LEAF AND ITS EXTRACTS ON RUMEN MICROBES, BIOHYDROGENATION AND METHANE PRODUCTION

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Chairman : Associate Professor Goh Yong Meng, PhD
Faculty : Veterinary Medicine

Methane (CH_4) emission from enteric fermentation is one of the causes of climate changes and global warming. Rumen biohydrogenation (BH) is also detrimental to human health because of increasing the risk of cardiovascular diseases. The effect of increasing inclusion levels of papaya leaf (PL), PL extract (PLE) and PL with different solvent fractions (PLFs) on biohydrogenation (BH) of polyunsaturated fatty acids (PUFA), microbial quantification and fermentation characteristics were studied.

For three *in vitro* studies, PL at different concentrations: 0 (control; CON, 50% alfalfa hay (AH) + 50% concentrate), 15% of AH in substrate replaced by PL (18.75 mg /250 mg dry matter, DM), 25% of AH in substrate replaced by PL (31.25 mg/250 mg DM) and 50% of AH in substrate replaced by PL (62.50 mg/250 mg DM) were mixed with 30 ml of buffered rumen fluid and were incubated for 24 h. CH_4 production (mL/250mg DM) declined at a decreasing rate (linear, $p = 0.03$) with increasing levels of PL. Supplementation of the diet with PL significantly ($P<0.05$) decreased the rate of BH of LA and C18-PUFA after 24 h of incubation, especially at the higher inclusion rate, resulting in an increased production of rumen BH intermediates such as conjugated linoleic acid (CLA), whereas saturated fatty acid (SFA) decreased in the rumen fluid after 24 h of incubation. Total methanogens ($P<0.05$) were lower in MPL (-12%) and HPL (-16%) compared to CON. Moreover, there were increases in total bacterial population (HPL: +5%) and *Butyrivibro fibrisolvens* (MPL: +20; HPL: +23%) among PL treatment groups compared to the CON group.

PLE at different concentrations with no addition of PLE (CON, 0), 5 mg /250mg DM (low leaf extract: LLE), 10 mg /250mg DM (medium leaf extract: MLE) and 15 mg /250mg DM (high leaf extract: HLE) was mixed with 30 ml of buffered rumen fluid and were incubated for 24 h. CH_4 production decreased ($P<0.05$) with increasing levels of PLE. Supplementation of the diet with PLE significantly ($P<0.05$) decreased the rate of BH of oleic acid (OA), linoleic acid (LA), α-linolenic acid (LNA) and C18-PUFA compared to CON after 24 h incubation resulting in increased concentrations of BH

intermediates such as vaccenic acid (VA), rumenic acid (RA) and t10c12 CLA. Total protozoa and methanogen population in HLE decreased ($P<0.05$) compared to CON, but the total bacteria and *Butyrivibrio fibrisolvens* population were higher ($P<0.05$) in CON compared to the PLE treatment groups.

Different solvent fractions of PL (PLFs; Hexane fraction, Chloroform fraction, Ethyl acetate fraction, Butanol fraction and water fraction) at a concentration of CON with no PLFs (0) and 15mg /250 mg DM which were equivalent to 0 and 60 g/kg DM, respectively were mixed with 30 mL of buffered rumen fluid and were incubated for 24 h. CH_4 production was highest ($P<0.05$) for CON (7.65) and lowest for chloroform fraction (5.41) compared to other PLFs at 24 h of incubation. Supplementation of the diet with PLFs significantly ($P<0.05$) decreased the rate of BH of OA, LA and LNA compared to CON after 24 h of incubation. The chloroform fraction had the lowest BH of OA (45.01%) and LA (54.65%) among treatments. Total protozoa and total methanogen populations in PLFs decreased ($P<0.05$) compared to CON, but the population of *Butyrivibrio fibrisolvens* was higher ($P<0.05$) in CON compared to PLFs treatment groups. These *in vitro* results indicate that PL, PLE and PLFs supplementation as the substrate decreased BH of PUFA, inhibited rumen methanogenesis without adversely affecting other rumen fermentation characteristics in rumen fermentation liquid.

For *in vivo* study, four fistulated adult Kajang cross-bred goats (39.0 ± 0.75 kg mean ± standard error) were used in a cross-over design study to test the effect of PL on rumen fermentation characteristics, rumen microbial population and FA composition of rumen fluid obtained at different times of sampling (0, 2, 4, 6 and 8 h). Goats were fed twice daily to the following diets: (CON, 50 % concentrate + 50% AH), 25% AH in basal diet replaced by PL (MPL), and 50% AH in basal diet replaced by PL (HPL). Rumen samples were collected at the end of the experiment (3rd weeks). Supplementation of the diet with PL significantly ($P<0.05$) decreased the rate of rumen BH of LA after different hours of feeding at the higher inclusion of PL (HPL), resulting in an increased concentration of rumen intermediate products such as CLA e.g. t10 c12, whereas SFA and stearic acid (SA) decreased ($P<0.05$) in the rumen fluid and blood plasma, respectively. The concentration of LNA was higher ($P<0.05$) in the blood of goats fed PL especially HPL, compared to the goats receiving only the basal diet with no PL inclusion (CON). The *Butyrivibrio fibrisolvens* population as determined by real-time PCR at the end of the feeding trial was higher ($P<0.05$) for MPL (+8%) and HPL (+16 %) in the rumen of goats compared to the CON. Methanogenic bacteria (log_{10}cell/L) decreased ($P<0.05$) in PL treatment groups.

In summary, this research revealed that supplementation of diets with different levels of PL even as a whole or as the extract (PLE and PLFs) plays an important role in affecting rumen microbial fermentation characteristics resulting in a reduction of CH_4 production (*in vitro*) and BH of FA (*in vitro* and *in vivo*).

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

PENILAIAN POLIFENOL YANG TERDAPAT DALAM DAUN POKOK BETIK *(CARICA PAPAYA* L.*)* DAN EKSTRAK DALAM RUMEN MIKROB, RUMEN BIOHYDROGENATIONDAN RUMEN PENGHASILANMETANA

Oleh

SAEID JAFARI

Mac 2016

Pengerusi : Profesor Madya. Goh Yong Meng, PhD
Fakulti : Perubatan Veterinar

Daun betik (PL) telah menjadi makanan haiwan yang berkhasiat dan mengandungi kepelbagaian spektrum metabolit sekunder. Kesan daripada peningkatan tahap kemasukan PL, ekstrak metanol PL (PLE) dan PL dengan pecahan pelarut yang berbeza (PLFs) di biohydrogenation (BH) asid lemak poli tak tepu (PUFA), kuantifikasi dan penapaian ciri-ciri mikrob telah dikaji.

Dalam kajian vitro, PL pada kepekatan yang berbeza PL: 0 (control; CON, 50% alfalfa hay (AH) + 50% pekat), 15% daripada AH dalam substrat digantikan dengan PL (18.75 mg / 250 mg bahan kering, DM), 25% daripada AH dalam substrat digantikan dengan PL (31.25 mg / 250 mg PTT) dan 50% daripada AH dalam substrat digantikan dengan PL (62.50 mg / 250 mg PTT) telah dicampur dengan 30 ml larutan cecair rumen dan dieram selama 24 jam . Pengeluaran metana (CH4) (mL / 250mg DM) merosot pada kadar yang berkurangan (linear P = 0.03) dengan tahap peningkatan PL.

Suplemen diet dengan PL ketara ($P<0.05$) mengurangkan kadar BH LA dan C18-PUFA selepas 24 jam pengeraman, terutama pada kadar kemasukan yang lebih tinggi, menyebabkan peningkatan penghasilan perantaraan rumen BH seperti asid linoleik (PTK), manakala asid lemak tepu (SFA) menurun dalam cecair rumen selepas 24 jam pengeraman. Jumlah methanogens ($P<0.05$) adalah lebih rendah pada MPL (-12%) dan HPL (-16%) berbanding dengan CON. Selain itu, terdapat peningkatan dalam jumlah bilangan bakteria (HPL: + 5%) dan fibrisolvens Butyrivibro (MPL: 20; HPL: + 23%) di kalangan kumpulan rawatan PL berbanding dengan kumpulan CON itu. PLE pada kepekatan yang berbeza tanpa penambahan PLE (CON, 0), 5 mg / DM 250mg (ekstrak daun rendah LLE), 10 mg / 250mg DM (ekstrak daun sederhana: MLE) dan 15 mg / 250mg DM (ekstrak daun tinggi : HLE) telah dicampur dengan 30 mL larutan cecair rumen dan dieram selama 24 jam. Pengeluaran CH4 menurun ($P<0.05$) dengan peningkatan tahap PLE. Suplemen diet dengan PLE ketara ($P<0.05$) mengurangkan kadar BH asid oleik (OA), asid linoleik (LA), α-linolenik (LNA) dan C18-PUFA berbanding CON selepas 24 jam pengeraman mengakibatkan peningkatan kepekatan

perantaraan BH seperti asid vaccenic (VA), asid rumenic (RA) dan t 10 c 12 CLA. Jumlah protozoa dan populasi methanogen dalam HLE menurun (P<0.05) berbanding CON, tetapi jumlah bakteria dan populasi Butyrivibrio fibrisolvens adalah lebih tinggi (P<0.05) dalam CON berbanding dengan kumpulan rawatan PLE.

Pecahan yang berbeza pelarut PL (PLFs; pecahan heksana, pecahan Chloroform, pecahan Ethyl asetat, pecahan Butanol dan pecahan air) pada kepekatan CON tanpa PLFs (0) dan 15mg / 250 mg DM yang bersamaan dengan 0 dan 60 g / kg DM, masing-masing telah dicampur dengan 30 mL larutan cecair rumen dan dieram selama 24 jam. Antara rawatan PLFs, pecahan kloroform tidak jauh berbeza (P> 0.05) berbanding dengan rawatan tanpa penambahan PLFs (CON: 50% AH + 50% pekat) dari segi pengeluaran jumlah gas (mL) dan kadar pengeluaran gas (mL / h).

Pengeluaran CH4 adalah paling tinggi (P<0.05) bagi CON (7.65) dan terendah untuk pecahan kloroform (5.41) berbanding PLFs lain pada 24 jam pengeraman. Suplemen diet dengan PLFs ketara (P<0.05) mengurangkan kadar BH OA, LA dan LNA berbanding CON selepas 24 jam pengeraman. Pecahan kloroform mempunyai BH terendah OA (45.01%) dan LA (54,65%) di kalangan rawatan. Jumlah protozoa dan jumlah populasi methanogen dalam PLFs menurun (P<0.05) berbanding CON, tetapi populasi fibrisolvens Butyrivibrio adalah lebih tinggi (P<0.05) dalam CON berbanding kumpulan rawatan PLFs. Keputusan dalam vitro ini menunjukkan bahawa PL, PLE dan PLFs suplemen sebagai substrat menurun BH daripada PUFA, menghalang rumen methanogenesis tanpa menjejaskan ciri-ciri rumen penapaian lain dalam cecair rumen penapaian.

Dalam kajian vivo, empat ekor kambing dewasa yang difistula telah digunakan dalam kajian reka bentuk cross-over untuk menguji kesan PL kepada ciri-ciri rumen penapaian, populasi rumen mikrob dan komposisi FA cecair rumen diperolehi pada masa yang berlainan persampelan (0, 2, 4, 6 dan 8 jam). Kambing dengan berat badan (BW) dari (39.0 ± 0.75 kg min ± SE) telah diberi makan dua kali sehari pada pukul 8:00 dan 5:00 untuk diet yang berikut: (CON, 50% pekat + 50% Hijrah), 25% AH dalam diet basal digantikan dengan PL (MPL), dan 50% AH dalam diet basal digantikan dengan PL (HPL). Darah dan rumen sampel telah dikumpulkan pada akhir eksperimen (minggu ke-3). Keputusan kami menunjukkan bahawa pH cecair rumen adalah lebih rendah (P<0.05) dalam HPL berbanding CON terutama pada 0 dan 6 jam rumen persampelan. Suplemen diet dengan PL ketara (P<0.05) mengurangkan kadar rumen BH LA selepas waktu yang berbeza memberi makan pada kemasukan yang lebih tinggi PL (HPL), mengakibatkan peningkatan kepekatan produk perantaraan rumen seperti CLA contohnya t10 C12, manakala SFA dan asid stearik (SA) menurun (P<0.05) di dalam cecair rumen dan plasma darah, masing-masing. Kepekatan LNA adalah lebih tinggi (P<0.05) dalam membawa darah pada kambing jantan diberi makan PL terutama HPL, berbanding kambing hanya menerima diet basal tanpa kemasukan PL (CON). Populasi Butyrivibrio fibrisolvens seperti yang ditentukan oleh masa sebenar PCR pada akhir percubaan makan adalah lebih tinggi (P<0.05) bagi MPL (+ 8%) dan HPL (16%) dalam rumen kambing berbanding CON itu. Bakteria metanogen (log10cell / L) menurun (P<0.05) dalam kumpulan rawatan PL.

Secara ringkasnya, kajian mendapati suplemen diet dengan pelbagai peringkat PL walaupun secara keseluruhannya atau secara ekstrak (PLE dan PLFs) memainkan peranan yang penting dalam mempengaruhi fermentasi rumen mikrob yang menyebabkan penurunan pengeluaran CH_4 dan BH bagi FA.

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

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ADF	acid detergent fiber
ANOVA	analysis of variance
BH	biohydrogenation
^{o}C/min	degree centigrade per minute
CH_4	Methane
CLA	conjugated linoleic acid
CT	condensed tannin
DM	dry matter
FAME	fatty acid methyl esters
G	Gram
GLM	general linear model
GHG	green house gas
H	Hour
HT	hydrolyzed tannin
Kcal	kilo calories
Kg	Kilogram
LA	linoleic acid
LNA	α-linolenic acid
Mm	Millimeter
MDA	Malondialdehyde
ME	metabolizable energy
Min	Minute
mmol/L	millimoles per liter
µmol/L	micromoles per liter
mg/L	milligrams per liter

Ml	Milliliter
MUFA	monounsaturated fatty acids / monoenoic fatty acids
NDF	neutral detergent fiber
OA	oleic acid
PL	papaya leaf
PLE	papaya leaf extract
PLFs	papaya leaf fractions
PSM	plant secondary metabolite
PUFA	polyunsaturated fatty acids
RA	rumenic acid
SA	stearic acid
SEM	standard error of mean
SFA	saturated fatty acids
UFA	unsaturated fatty acids
VA	vaccenic acid
VFA	volatile fatty acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Animals, particularly ruminants, produce methane (CH_4) from anaerobic fermentation in their gastrointestinal tracts as a pathway for the disposal of metabolic hydrogen produced during microbial metabolism (Jayanegara *et al.*, 2011). Methane accounts for 2-12% loss of dietary gross energy in ruminants and is a potent greenhouse gas (GHG) with a global warming potential 23 times higher than that of carbon dioxide (CO_2) in trapping the heat (Bhatta *et al.*, 2013; IPCC, 2001). Therefore, reducing ruminal CH_4 not only improves the efficiency of nutrient utilization but also helps to protect the environment from global warming. Manipulating the rumen microbial ecosystem to reduce CH_4 emission and nitrogen excretion by ruminants to improve their performance is an important issue for animal nutritionists. There is a need for identifying feed additives with the potential to modify rumen fermentation for enhancing the efficiency of utilization of feed energy while decreasing rumen methanogenesis (Bhatta *et al*., 2012).

The production of healthy food is an important issue in current ruminant nutrition research. The conversion of dietary polyunsaturated fatty acid (PUFA) to saturated fatty acid (SFA) by ruminants, which is called rumen biohydrogenation (BH) has important health implications for human health. In fact, cardiovascular diseases due to using animal products with high SFA are known to be one of the important causes of death in human populations worldwide (WHO, 2011). Moreover, the transformation of PUFA to SFA results in an increased accumulation of several hydrogenation intermediates in ruminant meat and milk, such as rumenic acid (RA) and vaccenic acid (VA), which are well known for their anti-carcinogenic, anti-atherogenic and anti-oxidative health promoting properties (Durmic *et al.*, 2008). Increased conjugated linoleic acid (CLA) levels in ruminant-derived food products can be achieved by nutritional and managerial practices that facilitate the higher forestomach output of CLA and VA (Chilliard *et al*., 2007).

Feed additives ranging from antibiotics, CH_4 inhibitors and defaunating agents have been used for years in ruminant nutrition to improve performance and decrease CH_4 production (Benchaar *et al*., 2008). However, negative consequences of these feed additives have encountered nutritionists with big problems and have stimulated them to search for the safer natural alternatives that would be useful in organic livestock farming. Moreover, the scarcity of feed resources often imposes a major challenge to the development of animal production in the tropics and subtropics. For years ago, it has been shown that tree leaves containing bioactive compounds are the alternative feed source for ruminant and can help to minimize the wide gap between availability and supply of nutrients, and improve the animal growth and productivity (Wanapat *et al.*,2012). On the other hand, the increasing cost of feeds and climatic problem associated with low production of cereals, which are key in keeping consistent supply

of concentrate used to feed ruminants, may reduce the applicability of using concentrate as a supplement for ruminant. In many arid and semi arid areas of the tropics, the available alternative would be to supplement the ruminant diet using shrubs and tree leaves as they have been proven to be rich in crude protein as compared to straws and grasses. Tree leaves were found to be rich in protein, soluble carbohydrates, minerals and vitamins, and showed great potential as an alternative feed resource (Bakshi and Wadhwa, 2007). Plants containing bioactive compounds have shown to be more effective in reducing rumen methanogenesis and rumen BH (Jayanegara *et al*., 2011). Plant extracts containing bioactive compounds such as essential oils, tannins, saponins, and flavonoids have been shown to improve rumen metabolism, to decrease the methanogenesis and protein degradation in the rumen and to increase the microbial protein production (Patra and Saxena 2011; Wallace *et al*., 2004). Plant extracts have also been used for centuries for various purposes such as traditional medicine and food preservatives due to their antimicrobial properties (Busquet *et al*., 2006; Davidson and Naidu, 2000). Jayanegara *et al*. (2011) showed that phenols from tropical forages were able to modulate BH and methanogenesis processes simultaneously and both towards a desirable direction. The papaya tree is a tree-like plant, a member of the small family *Caricaceae* and widely cultivated for its edible fruits and can be found in all tropical countries and many sub-tropical regions of the world (Canini *et al*., 2007). Parts of this plant are used for special purposes. Papaya leaf has been shown as a nutrient source and also as the bioactive compound (presence of phenolic acids) (Ayoola and Adeyeye 2010; Canini *et al*., 2007). Papaya leaf exhibited the highest phenolic content among other parts of the papaya such as unripe papaya fruit, ripe papaya fruit and seed (Maisareh *et al*., 2014). Presence of phytochemical contents and antimicrobial activities is also confirmed in PL extracts by ethanol, methanol, Ethyl acetate, Petroleum ether, hexane and water solvents (Baskaran *et al*., 2012). By considering the negative effect of rumen methanogenesis and rumen BH on the environment and health of consumers, respectively, and necessity of a safe feed alternative, the current study designed to address these issues in ruminant with the following hypotheses and objectives:

1.2 Hypothesis Statements

1. Incorporation of diets with PL can increase blood antioxidant activity in the goat *in vivo*.
2. Incorporation of diets with PL, PL extracts and PL fractions will not have any adverse effects on the rumen fermentation and rumen microbial population in the rumen fluid obtained from goat *in vitro*.
3. Incorporation of diets with PL, PL extracts and PL fractions will reduce the rumen BH in the goat.

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APPENDIX

Plate 1: Separation of papaya leaf from the stem

Plate 2. Rumen fistulated goat used for *in vitro*and *in vivo* studies.

BIODATA OF STUDENT

Saeid Jafari was born in Tehran, capital of Iran on 7th September 1984. He completed his primary, secondary and high school education in Tehran. He pursued his B.Sc degree at the Faculty of Agriculture, Islamic Azad University of Saveh and graduated in 2007.

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Saeid Jafari, Goh Yong Meng,Mohamed Ali Rajion, Mahdi Ebrahimi. Effect of papaya *(Carica papaya)* leaf on gas production *in vitro* gas production technique.Proceedings of the 35thMalaysian Society for Animal Production (MSAP) Annual Conference. 4 - 6 th June, 2014, Sarawak, Malaysi, PP. 69-70.

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Saeid Jafari, Goh Yong Meng,Mohamed Ali Rajion, Mohammad Faseleh Jahromi,Mahdi Ebrahimi. Ruminal methanogenesis and biohydrogenation reduction potential of papaya *(Carica papaya)* leaf: an *in vitro* study. (Published)

Saeid Jafari, Goh Yong Meng,Mohamed Ali Rajion, Mohammad Faseleh Jahromi,Mahdi Ebrahimi.Papaya *(Carica papaya)* leaf methanolic extract modulates *in vitro* rumen methanogenesis and rumen biohydrogenation. (accepted).

Saeid Jafari, Goh Yong Meng, Mohamed Ali Rajion, Mohammad Faseleh Jahromi,Mahdi Ebrahimi. Polyphenol rich solvent fractions from papaya leaf can modulate rumen fermentation characteristics, rumen biohydrogenation and rumen microbial population *in vitro.* (Published).

Saeid Jafari, Goh Yong Meng,Mohamed Ali Rajion, Mohammad Faseleh Jahromi,Mahdi Ebrahimi. Papaya *(Carica papaya)* leaf as a possible natural alternative for modulating rumen fermentation characteristics and rumen microbial population in goats: an *in vivo* study (Submitted).

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