UNIVERSIDADE DE SÃO PAULO
CENTRO DE ENERGIA NUCLEAR NA AGRICULTURA

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Effect of tanniniferous plants and essential oils on methane emission in ruminants

Piracicaba
2012

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Versão revisada de acordo com a resolução CoPGr 6018 de 2011

Tese apresentada ao Centro de Energia Nuclear na Agricultura da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Área de Concentração: Energia Nuclear na Agricultura e no Ambiente

Orientador: Prof. Dr. Adibe Luiz Abdalla

Piracicaba
2012

Dados Internacionais de Catalogação na Publicação (CIP)
Seção Técnica de Biblioteca - CENA/USP

Abd El-Rahman, Yosra Ahmed Soltan

Effect of tanniniferous plants and essential oils on methane emission in ruminants / Yosra Ahmed Soltan Abd El-Rahman; orientador Adibe Luiz Abdalla. - - Piracicaba, 2012.

107 f.: il.

Tese (Doutorado – Programa de Pós-Graduação em Ciências. Área de Concentração: Energia Nuclear na Agricultura e no Ambiente) – Centro de Energia Nuclear na Agricultura da Universidade de São Paulo.

1. Agricultura sustentável 2. Digestibilidade 3. Efeito estufa 4 Fermentação 5. Metabólitos secundários 6. Óleos essenciais 7. Ruminantes I. Título

CDU (591.133.1 + 633.87) : 504.7

أُهدي هذه الرساله إلي

أرواح شهداء حرية و كرامة مصر

ACKNOWLEDGEMENTS

First of all, my obedience and deepest thanks to Allah, the most gracious, the most beneficent and merciful; for the help and guidance to achieve goals and to make them possible.

I wish to express my great independence grateful to Prof. Dr. Adibe Luiz Abdalla from Centre for Nuclear Energy in Agriculture, University of Sao Paulo, Brazil. It was my great pride to complete this work under your supervision. I am very grateful for your confidence, attention, close kind help, support, learning experience and encouragement. You were very efficient useful guide and I am really thankful.

To Prof. Dr. Sobhy Mohamed Abdalla Sallam from Faculty of Agriculture, Alexandria University, Egypt, who introduced me to Prof. Dr Adibe and supported me to study abroad, really grateful thanks for your kind advice, supporting, attention, scientific discussion, cooperation and continual guidance and to greatly improve my work.

To Prof. Dr. Severino Matias de Alencar from College of Agriculture" Luiz de Queiroz", University of Sao Paulo, Brazil, for his support, important suggestions in the laboratory analysis and valiable suggestions in the qualification exam. Really thank you.

To Prof. Dr. Helder Louvandini, from Centre for Nuclear Energy in Agriculture, University of Sao Paulo, Brazil, for his encouragement and support the foreign students, laboratory facilities and scientific discussion. Really thank you.

To Prof. Dr. Vânia Rodrigues Vasconcelos, from Center of Agricultural Science, Federal University of Piaui. Thank you for your essential support and help in learning Portuguese.

To Prof. Dr. Elisabete Aparecida Fernandes de Nadai, and Prof. Dr. Adriana Pinheiro Martinelli, Coordinators of Post Graduation Programm at the Centre for Nuclear Energy in Agriculture, for all the support during the doctorate programm. Your support was essential to studying here at CENA and for this thesis to be finished. I am really grateful for your attention and support. I am also grateful for all the CPG staff for their support.

To Prof. Dr. Antonio Vargas de Oliveira Figueira, dean of the Center for Nuclear Energy in Agriculture, University of Sao Paulo Piracicaba, Brazil. I am really grateful for your support to complete my PhD at CENA.

To Prof. Dr. Jacinta D. F. Gomes for your kind support, friendship and helping during the gas production analysis, really thank you so much.

To Ms. Marilia R.G. Henyei, I am really thankful for your helping in learning Portuguese and most of all, for your personal support from the first day in Brazil that allowed me to have extraordinary life indeed. Thanks for your support in reviewing my qualification exam and of the thesis format. You were very efficient and kind and I am really thankful.

I am thankful for the technical support of Ms. Regina S.R. Peçanha, Mr. Lécio A. Castilho and Mr. Everaldo E. M. dos Santos and all the under graduate student trainees of Laboratory of Animal Nutrition of CENA for their collaboration in my experiments and the laboratorial analysis. Really thank you.

To my husband Amr Salah Morsy for your love, encouragement to study abroad and your unconditional help, support, participation in my experiments and all the analysis. I am really thankful for my family, my mother, father, sister, brother and my son Youssef because without your love, support, kind, encouragement and advice it was too hard to complete this work. I am very grateful for you and very a proud to be one of you.

To all friends and colleagues that helped in this work. To my friends, Ronaldo Carlos Lucas, Hani Mohamed Elzaiat, Kheir El-Din Moustafa El-Azrak, Adibe Luiz Abdalla Filho, Rafael Canonenco Araujo, Lília Raquel Fé, Edvania Pontes, Patricia Pimentel dos Santos, Patricia Barbosa de Godoy, Bernardo Berenchtein, Alessandra Romero, Aline Campeche, Tiago do Prado Paim, Alexander M. Greiling, Ana Flávia Rebram, Vitor Bonatto Guerrini. For all the help during the experiments, the labaroatory work, always supporting me, important discussions and for Portuguese support. Really without your support, this work hardly would be done.

I am grateful also to the Third World of Academic Science (TWAS) and Brazilian National Council for Scientific and Technological Development (CNPq) for financial support.
This thesis could not be finished without support of god, professors, my family, my friends, and all the persons above, and I am really thankful to all.

Home is where your memories are,
It's where your secrets are kept.
Home is where you're happiest,
Yet home is where you've wept.

Home is where your parents live,
It's where you were raised.
Home is where you've been scolded most,
Yet home is where you're praised.

Home is where you make it,
It's where you believe the myth,
That home is when you're happy,
Just to love the people you're with.

Eric Brock

RESUMO

ABD EL-RAHMAN, Y. A. S. **Efeito de plantas taniníferas e óleos essenciais sobre a emissão de metano por ruminantes**. 2012. 107 f. Tese (Doutorado) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2012.

Taninos e óleos essenciais são metabólitos secundários que podem ser utilizados como modificadores naturais da fermentação ruminal para reduzir a emissão de metano (CH_4) de ruminantes. Para estudar a aplicação de plantas ricas em taninos oriundas do Egito e do Brasil, bem como óelos essencias disponíveis no comércio internacional, três estudos foram conduzidos no Laboratório de Nutrição Animal do Centro de Energia Nuclear na Agricultura da Universidade de São Paulo, Piracicaba. O primeiro estudo teve como objetivo investigar o potencial das plantas taniníferas prosopis (*Prosopis juliflora*), acácia (*Acasia saligna*), atriplex (*Atriplex halimus*) e leucena (*Leucaena leucocephala*) em ensaio *in vitro* de produção de gás, avaliando o potencial metanogênico, a degradabilidade ruminal da proteína e a digestibilidade da proteína pós-ruminal, em comparação com feno de Tífton (*Cynodon spp.*) como alimento sem tanino. O *ranking* das plantas de acordo com seu potencial de redução de CH_4 com base na matéria orgânica verdadeiramente degradada (MODV) foi acácia> leucena> atríplex> prosopis. Prosopis e leucena apresentaram maior (P=0,002) produção de propionato (C3) com diminuição (P=0,004) correspondente na relação acetato:propionato (C2/C3). Acácia e leucena apresentaram menor (P=0,0002) concentração de NH_3-N associada com a diminuição na degradabilidade ruminal da proteína. No entanto, a leucena mostrou maior (P<0,0001) digestibilidade da proteína intestinal que a acácia. O objetivo do segundo estudo foi avaliar *in vitro* o potencial dos óleos essenciais carvacrol (CAR) e eugenol (EUG), nas doses 5, 10 e 20 (CAR) e 10, 20 e 30 (EUG) µl/75ml de fluido de cultura, como alternativa de modificadores naturais da fermentação ruminal em comparação com a Monensina (MON) (3µM/75ml de fluido de cultura) como controle positivo. CAR10 e EUG20 apresentaram similaridade na CH_4 e MODV comparado com MON, no entanto foram diferentes (P<0.05) no perfil de AGV, onde MON aumentou a concentração de C3 e diminuiu C2/C3, mas ambos CAR10 e EUG20 aumentaram (P<0,0001) as concentrações de butirato. Leucena foi selecionada no terceiro estudo para avaliação *in vivo* da atividade biológica de taninos na digestibilidade aparente, balanço de nitrogênio, fermentação ruminal e emissão de CH_4. Seis ovinos Santa Inês adultos, canulados no rúmen (70±2,5kg) foram individualmente divididos em três dietas experimentais em delineamento quadrado latino duplo (3tratamentos, 3períodos, 6animais). A dieta controle (CNTRL), contendo feno de Tífton (70%), farelo de soja (21%) e milho (9%). A dieta (LEUC), contendo leucena (123 e 8,8g/kg MS taninos totais e taninos condensados, respectivamente), consistiu na dieta controle, tendo 50% do feno de Tífton substituído pela leucena. A dieta LPEG constituiu da dieta LEUC mais a adição de 20g/dia/animal de polietileno glicol (PEG). Dietas contendo leucena aumentaram (P=0,009) a ingestão de matéria seca e nitrogênio (P=0,005) em comparação com CNTRL, enquanto não houve diferenças significativas para as digestibilidades aparentes das nutrientes mas a dieta LEUC diminuiu (P=0,0009) a digestibilidade da fibra em detergente ácido, amônia ruminal (P<0,0001). Dietas contendo leucena melhoraram (P=0.012) o balanço de nitrogênio e

diminuíram (P<0,0001) CH_4 bem como diminuíram (P<0,0001) C2/C3 em comparação com CNTRL. Estes estudos destacam o potencial das plantas taniníferas e os óleos essenciais para modular a fermentação ruminal e reduzirem a emissão de CH_4.

Palavras-chave: Taninos. Leucaena. Carvacrol. Eugenol. Fermentação. Sustentabilidade.

ABSTRACT

ABD EL-RAHMAN, Y. A. S. **Effect of tanniniferous plants and essential oils on methane emission in ruminants**. 2012. 107 f. Tese (Doutorado)- Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2012.

Tannins and essential oils are secondary metabolites that may be used as natural modifiers of rumen fermentation to reduce the ruminants' methane (CH_4) emission. To study the application of tannin-rich plants from Egypt and Brazil, as well as essential oils that are available in international trade, three studies were conducted at Animal Nutrition Laboratory of Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, at Piracicaba, Brazil. The first study was aimed to assess the potential nutritive value of tanniniferous plants prosopis (*Prosopis juliflora*), acacia (*Acasia saligna*), atriplex (*Atriplex halimus*) and leucaena (*Leucaena leucocephala*) leaves *in vitro* gas production assay, evaluate the methanogenic activity, ruminal fermentation, degradability and post ruminal protein digestibility compared with Tifton hay (*Cynodon spp*.) as non tannin feed. The ranking of the plants according to their potential to reduce CH_4 based on organic matter truly degraded (OMTD) was acacia> leucaena > atriplex> prosopis. Prosopis and leucaena presented greater (P=0.002) propionate (C3) production with corresponding decrease (P=0.004) in the acetate:propionate ratio (C2/C3). Acacia and leucaena leaves showed lower (P=0.0002) NH_3-N concentration associated with the decline in protein ruminal degradability. However, leucaena showed greater (P<0.001) intestinal protein digestibility than acacia. The objective of the second study was to evaluate *in vitro* the potential of constituents of essential oils carvacrol (CAR) and eugenol (EUG) at doses of 5, 10 and 20 (CAR) and 10, 20 and 30 (EUG) µl/75ml of culture fluid, as a natural alternative to rumen microbial fermentation modifiers compared with monensin (MON) (3 µ M/75ml of culture fluid) as a positive control. CAR10 and EUG20 showed similarity (P>0.05) in reduction of CH_4 emission and OMTD compared with MON, but it had different (P<0.05) short chain fatty acids (SCFA) profile. Monensin increased C3 concentration and decreased C2/C3 ratio, but CAR10 and EUG20 increased (P<0.0001) concentrations of butyrate without effect on the total SCFA. Leucaena was selected in the third study to evaluate *in vivo* the tannins biological activity, total apparent digestibility, nitrogen balance, rumen fermentation and CH_4 emission. Six adult rumen cannulated Santa Inês sheep (70±2.5 kg) were individually divided into three experimental diets in a double Latin square design (3 treatments, 3 periods, 6 animals). In the control diet (CNTRL), animals received a basal diet containing Tifton hay (70%), soybean meal (21%) and ground maize (9%). The second diet contained leucaena (LEUC), (123 and 8.8 g/kg DM of total tannins and condensed tannins respectively) replacing 50% of Tifton hay basal diet. The third diet (LPEG), polyethylene glycol was supplemented at rate of 20g/day/animal. Leucaena-containing diets increased intake of dry matter (P=0.009) and nitrogen (P=0.005) compared with CNTRL, while there were no significant differences among all diets for the nutrients apparent digestibility except for acid detergent fiber (ADF) was reduced (P=0.0009) by LEUC. Leucaena-containing diet improved (P=0.012) the nitrogen balance and reduced

(*P*<0.001) rumen ammonia concentration. Leucaena-containing diets decreased (*P*<0.001) CH_4 emission as well as reduced (*P*<0.001) C2/C3 ratio compared to CNTRL. These studies highlight the potential of tanniniferous plants and the essential oils active components to modulate the rumen fermentation and to reduce CH_4 emission in ruminants.

Keywords: Tannins. Leucaena. Carvacrol. Eugenol. Fermentation. Sustainability.

الملخص العربي

يسرا أحمد سلطان عبد الرحمن . تأثير النباتات المحتوية علي التانينات و الزيوت الطيارة (معملياً و حقلياً) علي إنتاج الميثان في المجترات ٢٠١٢. ١٠٧ صفحه . رسالة دكتوراه- مركز الطاقه النووي الزراعي - جامعةساوبولو- بيراسيكابا- البرازيل ٢٠١٢.

تعتبر التانينات و الزيوت الطيارة من النواتج الثانويه الميتابولزميه في النباتات و التي إستخدمت لتعديل بيئة كرش المجترات لخفض إنتاج الميثان .لذلك أجريت ثلاثة دراسات, الأولي كانت تهدف إلي تقييم معملي لبعض النباتات المحتوية علي التانينات مثل البروسوبس(*Prosopis juliflora*), الأكاسيا (*Acasia saligna*) , القطف (*Atriplex halimus*) و الليوسينا (*Leucaena leucocephala*)لإنتاج الغاز و الميثان و عمليات التخمر بالكرش و كذلك تحلل البروتين بالكرش و ما بعد الكرش معملياً و ذلك بالمقارنه بدريس التيفتون (*Cynodon spp.*) كمادة علف خاليه من التانينات. و أوضحت النتائج أن ترتيب النباتات تبعاً لخفض إنتاج الميثان كالتالي: الأكاسيا > الليوسينا> القطف> البروسوبس (٣٧,٩ ، ٣٥,٣، ٢٦,٣ و٢٦,٠٪ إعتماداً علي الهدم الحقيقي للمادة العضوية علي التوالي) وقد أظهر كل من نبات البروسوبس و الليوسينا زيادة في إنتاج البروبيونك بمعنويه =٠,٠٠٢مع خفض مماثل لنسبة الأسيتك:البروبيونك بمعنوية =٠,٠٠٤. بينما أقل قيم للماده العضويه المهضومه حقيقياً كانت للأكاسيا ودريس التيفتون بمعنويه <٠,٠٠١بالمقارنه بكل النباتات الأخري. وأظهرت كل من الأكاسيا و الليوسينا أقل قيم لتركيزات الأمونيا بالكرش بمعنويه =٠,٠٠٢و هذا الإنخفاض متلازم مع الإنخفاض في تحلل البروتين بالكرش,هذا علي الرغم من أن الليوسينا أظهرت معدل هضم للبروتين في الأمعاء أعلي من الأكاسيا بمعنويه <٠,٠٠١. الدراسة الثانية أجُريت بهدف إختبار المكونات الفعالة الأكثر شيوعاً للزيوت الطيارة و هي الكارفاكرول و الإيجانول بتركيز (٥ , ١٠ , ٢٠ ميكروليتر لكل ٧٥مل من مخلوط سائل الكرش والمحلول المنظم) و (١٠,٢٠,٣٠ ميكروليترلكل ٧٥مل من مخلوط سائل الكرش والمحلول المنظم) علي التوالي كبدائل طبيعية لتعديل التخمر بالكرش معملياً بالمقارنة بالمونينسن (٣ميكرو مول لكل ٧٥ ملي من سائل الكرش والمحلول المنظم). كل من الكارفاكرول و الإيجانول بتركيز ١٠و ٢٠ميكروليتر علي التوالي أظهرت تشابه في إنتاج الغاز والميثان و هدم المادة العضوية بالكرش مع المونينسن علي الرغم من الاختلاف في مسارات الأحماض الدهنيه قصيرة السلسلة حيث أن المونينسن أدي إلي زيادة البروبيونك و خفض نسبة الأسيتك:البروبيونك بمعنوية <٠,٠٠١لكن كل من الكارفاكرول و الإيجانول أدي الي زيادة البيوتيرك بدون إختلاف في المجموع الكلي للأحماض الدهنيه قصيرة السلسلة. الليوسينا تم إختيارها في الدراسه الثالثة للتقيم الحقلي للنشاط البيولوجي لمحتواها من التانينات علي الهضم الظاهري و الإتزان النيتروجيني و تخمرات الكرش و إنتاج الميثان. تمت الدراسة علي 6 كباش من سلالة سانتا إيناس (بمتوسط وزن ٧٠ كجم) و قسمت إلي ثلاثة مجاميع مستخدماً المربع اللاتيني (٣حيوانات و٣ معاملات و ٣ فترات) كالتالي: المجموعة الأولي كانت تتغذي علي العليقة الأساسية فقط و المجموعة الثانية كانت تتغذي علي عليقة الليوسينا (١٢٣و ٨,٨جرام / كجم ماده جافه تانين كلي و مكثف علي التوالي) و التي تتكون من العليقة الأساسية و لكن تم إستبدال ٥٠٪ من دريس التيفتون بأوراق الليوسينا بينما المجموعة الثالثة كانت تتغذي علي عليقة الليوسينا مضاف إليها ٢٠جرام بولي إيثلين جليكول لكل حيوان كل يوم. أظهرت علائقتي الليوسينا(المجموعه الثانية و الثالثة) إلي زيادة للمأكول من الماده الجافه بمعنوية =٠,٠٠٩ و النتروجين بمعنوية =٠,٠٠٥١ بالمقارنة بالمجموعة الأولي المقارنة بينما لا توجد إختلافات معنوية لهضم كل من الماده الجافة و العضوية والبروتين والألياف المقاومة للذوبان في المحاليل المتعادلة و الدهن و لكن مجموعة الليوسينا بدون اضافة البولي إيثلين

جليكول أدت إلي خفض كل من هضم الألياف المقاومة للذوبان في المحاليل الحامضية بمعنويه =٠,٠٠٩و تركيز الأمونيا بالكرش بمعنوية <.كما أدت عليقتي الليوسينا إلي تحسين الميزان الازوتي بمعنويه = ٠,٠١٢ و خفض معنوي<٠,٠٠١لإنتاج الميثان المحسوب علي أساس المهضوم من المادة العضوية كما أدت إلي خفض نسبة الأسيتك:البروبيونك بالمقارنة بالمجموعة المقارنة. وتشيرنتائج هذه الدراسات إلي كفاءة كل من النباتات المحتوية علي التانينات و الزيوت الطيارة لتعديل بيئة الكرش تجاه مسارات تقليل الميثان .

مفاتيح الكلمات : التانينات. اليوسينا. الكارفاكرول. الايجانول. التخمرات. الاستفاده.

ABBREVIATIONS

ADF	Acid detergent fiber
BCVFA	Branched chain volatile fatty acids
C2	Acetic acid
C2:C3	Acetate: propionate ratio
C3	Propionic acid
C4	Butyric acid
C5	Valeric acid
CAR	Carvacrol
CENA	Centre for Nuclear Energy in Agriculture
CH_4	Methane
CP	Crud protein
CT	Condensed tannins
DM	Dry matter
EUG	Eugenol
GP	Gas production
IPD	Intestinal protein digestibility
NDF	Neutral detergent fiber
OM	Organic matter
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
SCFA	Short chain fatty acids
TDOM	Truly degraded organic matter
TP	Total phenols
TT	Total tannins

SUMMARY

1 INTRODUÇÃO

O conhecimento dos processos fermentativos no rúmen é uma importante ferramenta na nutrição de ruminantes. A fermentação ruminal, característica distintiva entre o animal hospedeiro e a microflora no rúmen, confere aos ruminantes várias vantagens nos processos digestivos e metabólicos, as quais não estão presentes nos animais não ruminantes. No entanto, a fermentação ruminal da dieta resulta em excesso na produção de hidrogênio que necessita ser removido do rúmen para manutenção eficiente do processo de fermentação e crescimento microbiano (VAN ZIJDERVELD et al., 2010). A fermentação ruminal também resultar em ineficiências de degradação da proteína dietética pelos microorganismos proteolíticos no rúmen (PATRA; SAXENA, 2011).

Em geral, o hidrogênio é removido através da atividade das Archaea metanogênicas que reduzem o dióxido de carbono com hidrogênio para gerar metano (CH_4) e água pelo processo denominado metanogênese, que é o mecanismo favorecido pelo rúmen para evitar o acúmulo de hidrogênio durante a fermentação ruminal anaeróbica (BEAUCHEMIN et al., 2008).

Recentemente, houve crescente interesse mundial na redução da emissão de CH_4 e na degradabilidade ruminal da proteína pelos ruminantes. Em primeiro lugar, porque CH_4 é um gás de efeito estufa, onde aproximadamente 15% das emissões globais são de CH_4 entérico produzido por ruminantes domésticos (IPCC, 2001). A produção desse metano depende do tipo de dieta, do nível de alimentação, e das características dos ruminantes tais como o tamanho, idade e espécie.

As emissões de CH_4 do rúmen representam uma perda significativa de energia da dieta que poderiam ser redirecionadas para a produção de leite ou carne (ECKARD et al., 2010). Por exemplo, a redução de 25% em emissões de CH_4 entérico poderia aumentar o ganho de peso corporal de bovinos em crescimento em cerca de 75 g/d (BRUINENBERG et al., 2002) ou na produção de leite em vacas leiteiras em 1L/d com base nos balanços de energia (NKRUMAH et al., 2006).

Em relação à utilização da proteína da dieta pelos ruminantes, a proteína solúvel que é degradada pelos microorganismos do rúmen resultam em níveis excedentes de amônia (20–35%) a qual é absorvida a partir do rúmen e excretada na urina (ULYATT et al., 1975), sendo um potencial poluente ambiental porque o nitrogênio urinário é, em grande parte, na forma de ureia, mais rapidamente hidrolisado em amônia e nitrificado para nitrato

(ECKARD et al., 2010). Esse nitrato pode contaminar a água subterrânea causando a poluição da água, e também é convertido em óxido nitroso, outro gás do efeito estufa, e representa cerca de 65% das ações antropogénicas globais nas emissaões de óxido nitroso (ECKARD et al., 2010).

Assim, diminuir a emissão de CH_4 e a degradação protéica ruminal pelos ruminantes tem como alvo os dois objetivos, reduzir as emissões globais de gases de efeito estufa e melhorar a eficiência da conversão alimentar para produção de leite ou carne.

Teoricamente, o objetivo de melhorar a eficiência de utilização da energia e de proteína no rúmen poderia ser conseguido através da competição entre diferentes populações microbianas ruminais que modificam o ambiente e aumentam ou inibem populações microbianas específicas (CALSAMIGLIA et al., 2007). Para Moss et al. (2000) isto pode ser conseguido através da estimulação das vias de fermentação para consumir hidrogênio e evitar o efeito negativo do aumento da pressão parcial do gás. Manipular a via da fermentação ruminal para produzirem propionato pode ser considerado como uma via competitiva para utilização de hidrogênio no rúmen que, consequentemente, reduz a emissão de CH_4 (LIN et al., 2011). Além disso, favoreceria a redução da degradação da proteína no rúmen diminuindo a produção de amonia e, subsequentemente, aumentando a quantidade de proteína digerida no intestino delgado elevando a produtividade dos ruminantes (PATRA; SAXENA, 2011).

Aditivos químicos de alimentação tais como antibióticos ionóforos, inibidores de metano e agentes defaunadores podem ser considerados como aditivos ideais pra induzirem alterações na fermentação ruminal para incrementar a eficiência de energia e metabolismo de nitrogênio (N) no rúmen, diminuindo assim os processos de desperdício (BEAUCHEMIN et al., 2008; ARAUJO et al., 2011).

No entanto, o uso desses aditivos químicos como promotores de crescimento tem sido questionado devido a sua ligação com o desenvolvimento de resistência em agentes patogénicos bacterianos e a ameaça potencial para a saúde humana (CALSAMIGLIA et al., 2007). Antibióticos e outros aditivos sintéticos utilizados na alimentação animal têm sido efetivamente proibidos pela União Europeia desde 2006 (Regulação 1831/2003/EC). Assim, estudos recentes têm-se interessado em avaliar alternativas para controlar as populações microbianas específicas para modular a fermentação no rúmen para a produção de menos metano e ou menor degradabilidade da proteína no rumen.

A utilização de metabólitos secundários de plantas como taninos, óleos essenciais, saponinas, flavonóides e outros metabólitos secundários como modificadores do rúmen parece ser uma abordagem melhor, uma vez que estes são produtos naturais que podem ser ambientalmente amigáveis e ter uma melhor aceitação junto aos consumidores de produtos animais (AGARWAL et al., 2009; ABDALLA et al., 2012; JETANA et al., 2011; THEODORIDOU et al., 2011; LIN et al., 2011).

A atividade anti-metanogênica de plantas que contêm tanino tem sido atribuída principalmente aos taninos condensados que afetam diretamente na metanogênese ruminal e indiretamente sobre a produção de hidrogênio devido à degradação dos alimentos de baixa qualidade ou através de seu efeito antiprotozoário (TAVENDALE et al., 2005). Mais além, é geralmente aceito que os taninos diminuem a taxa de degradação da proteína no rúmen principalmente devido à formação de complexos tanino-proteína (pH do rúmen) e consequente inibição do crescimento e atividades de populações de bactérias proteolíticas (PATRA, 2011; JETANA et al., 2011).

Nos últimos anos, tem sido mostrado que alguns óleos essenciais e os seus componentes ativos são capazes de modificar a fermentação ruminal, mudando os modos de produção dos ácidos graxos de cadeia curta, metabolismo de proteínas, ou ambos (KILIC et al., 2011; CHAVES et al., 2012). No entanto, tais efeitos são inconsistentes e dependem da probabilidade do componente ativo interagir com o alvo (bactérias), o que depende da concentração do componente ativo que pode variar amplamente dependendo da planta, condições de crescimento, ou métodos de processamento para extração de óleo (LIN et al., 2011; SALLAM et al., 2011). Uma maneira de evitar resultados inconsistentes é o uso de doses de componentes ativos dos óleos essenciais puros em vez do todo o óleo ou extrato (CALSAMIGLIA et al., 2007).

1.1 Hipótese

Usando o complexo tanino-nutriente naturalmente estabelecido nas plantas tropicais e dietas suplementadas com componentes mais ativos dos óleos essenciais, proporcionará uma oportunidade para estudar as propriedades anti-metanogênicas e ações sobre fermentação ruminal *in vitro* e *in vivo* destes metabólitos secundários naturais na dieta de ovinos.

1.2 Objectivos

1.2.1 Objetivo Geral

Estudar o efeito das plantas taninferas prosopis (*Prosopis juliflora),* acacia *(Acacia saligna),* atriplex *(Atriplex halimus)* e leucena *(Leucaena leucocephala*) e dos óleos essenciais carvacrol e eugenol na emissão de metano, fermentação ruminal, degradação pós-rúmen, digestibilidade dos nutrientes e balanço de nitrogênio pelos ovinos.

1.2.2 Os objetivos específicos

a) Caracterização química das plantas taniniferas quanto o valor nutricional e o potencial de utilização como suplementos de proteína em dietas para melhorar a qualidade da alimentação visando melhorar a produtividade de ruminantes em regiões tropicais;

b) Determinação *in vitro* do efeito destas plantas sobre a produção de CH_4, características de fermentação e digestibilidade da proteína pós-rúmen;

c) Seleção da melhor planta quanto ao valor nutricional, potenciais propriedades anti-metanogênicas, sem efeito adverso na degradabilidade ruminal para ser testada em ensios *in vivo*;

d) Avaliação *in vitro* dos componentes ativos dos óleos essenciais carvacrol e eugenol como modificadores naturais de fermentação no rúmen para diminuir as emissões de CH_4 em dietas de ovinos em comparação com a monensina, antibiótico ionóforo usado para diminuir a produção de CH_4 pelo rúmen;

e) Avaliação *in vivo* do efeito da substituição do feno de Tífton pela leucena e suplementação com polietilenoglicol (PEG) sobre emissão de metano entérico, fermentação ruminal, digestibilidade dos nutrientes e o balanço de nitrogênio em ovinos.

1.3 Desenvolvimento

Os resultados deste estudo são apresentados na forma de capítulos. Nos primeiros capítulos estão introdução e revisão da literatura quanto à produção de metano em ruminantes e a ação de taninos e óleos essenciais na metanogenese ruminal. Posteriormente são apresentados três experimentos desenvolvidos para estudar o efeito de plantas taniníferas egipcias (capítulo 3) e o carvacrol e eugenol (capítulo 4) sobre a fermentação ruminal *in vitro* e o efeito da adição de polietileno glicol na dieta de ovinos alimentados com *Leucena leucocephala* (capítulo 5). Como conclusão geral tem-se que a estratégia de mitigação de metano usando as plantas taniníferas e os óleos essenciais estudados mostraram potencial para suprimir a metanogênese *in vitro* e *in vivo*; no entanto, o objetivo global de reduzir os gases do efeito de estufa pode não ser cumprido a menos que a avaliação do ciclo de vida dessas estratégias seja avaliada.

2 INTRODUCTION

It is well known that rumen fermentation processes play a key role in ruminant nutrition, as it is this distinctive symbiotic feature between the host and the rumen microflora that lends the ruminant animal several advantages in digestive and metabolic processes over non-ruminants. However, diet rumen fermentation results in the production of excess hydrogen, which needs to be removed from the rumen for the fermentation process and microbial growth to continue efficiently (VAN ZIJDERVELD et al., 2010). Also it results in protein inefficiencies by rumen microorganisms proteolysis (PATRA; SAXENA, 2011).

In general, hydrogen is removed through the activity of methanogenic Archaea, which reduce carbon dioxide with hydrogen to generate methane (CH_4) and water by methanogenesis, which is the mechanism favoured by the rumen to avoid hydrogen accumulation during anaerobic rumen fermentation (BEAUCHEMIN et al., 2008).

Recently, there are growing worldwide interest in reducing CH_4 emission and rumen protein degradability by ruminants. Firstly, because CH_4 is a potent greenhouse gas, of which approximately 15% of the global emissions are from enteric CH_4 produced by domestic ruminants (IPCC, 2001). In addition to this methane depend on type of diet, level of feeding, and ruminant characteristics such as size, age, and species. Rumen CH_4 emissions represent a significant loss of dietary energy that could potentially be redirected towards the production of milk and meat (ECKARD et al., 2010). For instance, a 25% reduction in CH_4 emissions could increase body weight gain of growing cattle by approximately 75 g/d (BRUINENBERG et al., 2002) or milk production of dairy cows by 1 L/d based on the energy balances (NKRUMAH et al., 2006).

Secondly, diet soluble protein degraded by rumen microorganisms resulting in surplus levels of ammonia (20–35%), which is absorbed from the rumen and excreted in urine (ULYATT et al., 1975) which decrease beneficial environmentally because urinary nitrogen is largely in the form of urea, which is more rapidly hydrolysed to ammonia and nitrified to nitrate (ECKARD et al., 2010). The nitrate could leach into groundwater causing water pollution, and is also converted to nitrous oxide (a greenhouse gas) accounting for about 65% of global anthropogenic nitrous oxide emissions (ECKARD et al., 2010).

Thus, decreasing the CH_4 emission and rumen protein degradation from ruminant livestock is targeting both goals, to reduce global greenhouse gas emissions and as a means of improving feed conversion efficiency for milk or meat production.

Theoretically, the objective of improving the efficiency of energy and protein utilization in the rumen could be achieved by the competition among different rumen microbial populations that modify the environment and enhance or inhibit specific microbial populations (CALSAMIGLIA et al., 2007). This has been achieved through the stimulation of fermentation pathways to consume hydrogen to avoid the negative effect of the partial pressure increase of this gas (MOSS et al., 2000). Thus, manipulating rumen fermentation pathway to one that produce propionate can be considered as a competitive pathway for hydrogen use in the rumen that consequently reduce CH_4 emission (LIN et al., 2011). Also, enhancing the reduction of protein degradation in the rumen will decrease ammonia production and subsequently increase the quantity of protein digested in the small intestine can enhance the productivity of ruminant livestock (PATRA; SAXENA, 2011).

A number of chemical feed additives such as ionophore antibiotics methane inhibitors and defaunating agents can be considered as good additives as they induce changes in rumen fermentation leading to increased efficiency of energy and N metabolism in the rumen thereby decreasing wasteful processes (BEAUCHEMIN et al., 2008; ARAUJO et al., 2011).

However, the use of these chemical feed additives as growth promoters has been questioned because of their link with development of resistance in bacterial pathogens and the potential threat to human health (CALSAMIGLIA et al., 2007). Antibiotics and other synthetic additives used in animal feeding have been effectively banned by the European Union since 2006 (regulation 1831/2003/EC). Thus, recent studies have become interested in evaluating alternatives to control specific microbial populations to modulate rumen fermentation towards less methane production.

The use of plant secondary metabolites like tannins, essential oils, saponins, flavonoids and many other plant secondary metabolites as rumen modifiers seems to be a better approach since these are natural products that might be environmentally friendly and have better acceptance with the consumers (AGARWAL et al., 2009; ABDALLA et al., 2012; JETANA et al., 2011; THEODORIDOU et al., 2011; LIN et al., 2011; BHATTA et al., 2012).

The antimethanogenic activity of tannin containing plants has been attributed mainly to condensed tannins, which affect directly on ruminal methanogens and an indirectly way on hydrogen production due to degradation of fibrous feed or through their anti-protozoal effect (TAVENDALE et al., 2005). More addition, it is generally agreed that tannins decrease the rate of the rumen protein degradation mainly due to the formation of tannin–protein complexes in the rumen pH and inhibition of the growth and activities of proteolytic bacterial populations (PATRA, 2011; JETANA et al., 2011).

Recently, it has been shown that some essential oils and their active components are able to modify rumen fermentation by shifting short chain fatty acids production pathways, protein metabolism, or both (KILIC et al., 2011; CHAVES et al., 2012). However, such these effects are inconsistent and depends on the probability of the active component interacting with the target bacteria, which depends on the concentration of the active component which can vary widely depending in the cultivar, growing conditions, or processing methods for oil extraction (LIN et al., 2011, SALLAM et al., 2011). Thus one way to avoid inconsistent results is to use doses of pure active components of essential oils rather than the whole oil or extract (CALSAMIGLIA et al., 2007).

2.1 Hypothesis

Using tannin-nutrient complex naturally set in tropical plants and diets supplemented with most active essential oil components, will provide an opportunity to study the anti metanogenic properties and actions on rumen fermentation *in vitro* and *in vivo* of these natural plants secondary metabolites in sheep diets.

2.2 Objectives

2.2.1 General objective

To study the effect of the tanninferous plants e.g. prosopis (*Prosopis juliflora),* acacia *(Acacia saligna),* atriplex *(Atriplex halimus)* and leucaena *(Leucaena leucocephala*), and carvacrol and eugenol as the active components of essential oils on methane emission, rumen fermentation, post rumen degradation, total tract nutrients digestibility and nitrogen balance by sheep.

2.2.2 Specific objectives

a) Chemical characterization of the tanninferous plants for their nutritional value and their potential as protein diet supplements to improve the feed quality of ruminants that could help to improve productivity of ruminant livestock in tropical regions;

b) Determination the *in vitro* effect of these plants on CH_4 production, fermentation characteristics, rumen degradability and post rumen protein digestibility;

c) Selection of the most promising plant according its nutritional value, potential antimethanogenic properties without adverse effect in the rumen degradability to be tested *in vivo*;

d) Evaluation of the essential oils most active components (carvacrol and eugenol) as natural modifiers of rumen fermentation to reduce CH_4 emission in sheep diets *in vitro* compared with monensin as the ionophore antibiotic used for reducing CH_4 production;

e) *In vivo* evaluation of the effect of replacement of tífton hay by leucena, and supplementation with polyethylene glycol (PEG) on enteric CH_4 emission, ruminal fermentation, nutrient digestibility and nitrogen balance in sheep.

2.3 Development

The results of this study are presented in the form of chapters. In the first chapters are the introduction and the review of the literature on methane production in ruminants and the action of tannins and essential oils on rumen methanogenesis. Following there are presented three experiments designed to study the effect of Egyptian tanniniferous plants (chapter 3) and eugenol and carvacrol oils (chapter 4) on rumen fermentation *in vitro* and the effect of addition of polyethylene glycol in the diet of sheep fed with *Leucena leucocephala* (chapter 5). As general conclusion has been that the methane mitigation strategy using the tanniniferous plants and essential oils studied showed potential to suppress methanogenesis *in vitro* and *in vivo*. However, the overall objective of reducing the greenhouse gases might not be met unless evaluated the entire lifecycle of these strategies.

2.4 Literature Review

2.4.1 Ruminants' methane formation

In the anaerobic conditions prevailing in the rumen, the oxidation reactions required to obtain energy in the form of ATP release hydrogen (H_2) which is one of the major end products of rumen fermentation by protozoa, fungi and pure monocultures of some bacteria (MOSS et al., 2000). Hydrogen does not accumulate in the rumen because it is immediately used by methanogenic bacteria that belong to the domain Archaea, which fall within the *Euryarchaeota* phylum and form five orders; Methanobacteriales, Methanosarcinales, Methanomicrobiales, Methanococcales and Methanopyrales. They are responsible for

removing the H_2 produced during microbial fermentation by methane formation, which is the major way of H_2 elimination through the following reaction:

$$CO_2 + 4\ H_2 \longrightarrow CH_4 + 2\ H_2O \text{ (MOSS et al., 2000).}$$

The methanogenic utilization of H_2 that produced by other rumen microorganisms is known as interspecies hydrogen transfer (IHT) (MILLER, 1995) and allows reduced cofactors (such as NADH) in the H_2- producing organisms to be reoxidised and recycled.

Attwood and Mcsweeney (2008) reviewed many examples explaining such these relationship between methanogenic and the other rumen microorganisms. For instance, during IHT between the cellulose-degrading rumen bacteria (*Ruminococcus flavefaciens)* and *Methanobrevibacter ruminantium*, utilisation by the methanogen of H_2 produced by *R. flavefaciens* allows NADH to be reoxidised to NAD+ and shifts its fermentation towards acetate rather than succinate. Also, methanogens use H_2 produced by rumen fungi, and in doing so alter their fermentation patterns and enhance the activities of their extracellular hemicelluloses degrading enzymes. More addition, some methanogens form ecto- and endosymbiotic relationships with rumen protozoa in which they are presumed to utilise H_2 produced from protozoal hydrogenosomes to produce CH_4.

Thus, the removal of H_2 by methanogens has a profound effect not only in the end-products of microbial fermentation but also the extent of degradation of plant fibre that occurs in the rumen (ATTWOOD; MCSWEENEY, 2008). For that, decreasing methane production must be accompanied by providing an alternative hydrogen sink or by minimizing the proportion of H_2 released (e.g., altering SCFA proportions towards increased propionate) (CALSAMIGLIA et al., 2006; 2007).

2.4.2 What are tannins?

Tannins represent an important class of plant secondary metabolites and are produced by the plants in their intermediary metabolism. The term “tannin” refers to “tanning” or preservation of skins to create leather, and also tannins contribute to the astringency properties of many popular drinks, as tea and wine (WAGHORN, 2008). Chemically, tannins are polyphenolic compounds with varying molecular weights, and they have the ability to bind natural polymers such as proteins and carbohydrates due to the presence of a large

number of phenolic hydroxyl groups on their structure (MUELLER-HARVEY, 2006). Based on their molecular structure, tannins are classified as either hydrolysable tannins (HT) or condensed tannins (CT) (Figure 1).

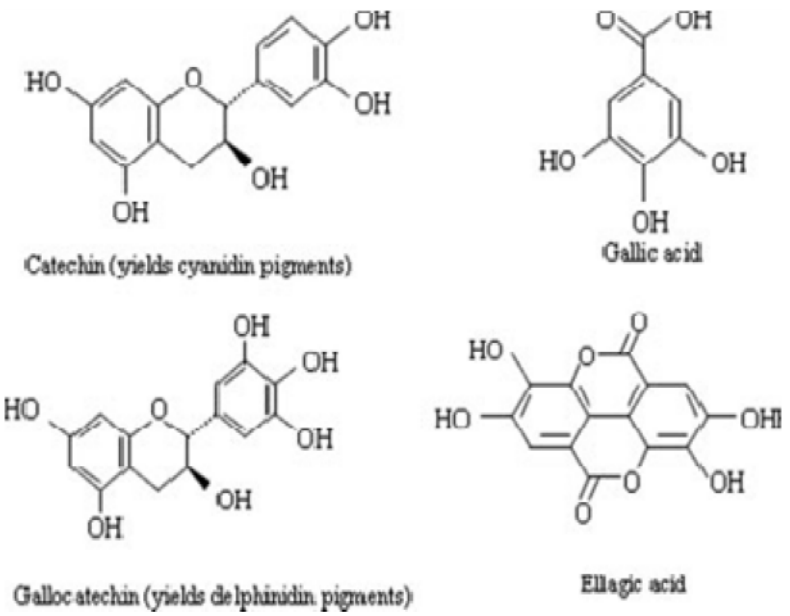

Figure 1 - Monomeric units of condensed (catechin and gallocatechin) and hydrolysable tannins (gallic and ellagic acid) (PATRA; SAXENA, 2011)

Hydrolysable tannins are polyesters of phenolic groups as gallic acid or ellagic acid with central core, such as glucose, glucitol, quinic acids, quercitol and shikimic acid, polymers of flavonoids). Hydrolysable tannins are susceptible to hydrolysis by acids, bases or esterases yielding polyol and the constituent phenolic acids (HASLAM, 1989).

Condensed tannins, or proanthocyanidins, are mainly polymers of the flavan-3-ol (epi) catechin and (epi) gallocatechin units, which are linked by C4–C8 and C4–C6 interflavonoid linkages. Many other monomers of CTs, e.g. profisetinidins, probinetidins and proguibortinidins, are also present. Quebracho tannins are largely profisetinidins. In procyanidins and prodelphinidins, C4–C8 and C4–C6 linkages with a ratio of about 3:1 are more common and the majority of tannins in these classes are of mixed stereochemistry with 2,3-*cis* to 2,3-*trans* ratios between 9:1 and 5:5. The number of monomeric units canvary and this determines the degree of polymerisation from di-, tri- and tetraflavonoids to higher oligomers. These can then produce an infinite variety of chemical structures, which in turn could produce different biological properties. The CTs are degraded to form monomeric

anthocyanidins (e.g. cyanidins and delphinidins) pigments upon treatment with acid butanol reaction. The CTs can react by hydrogen bonding with plant protein to form stable and insoluble CT–protein complexes at pH 3.5–7.0, which dissociate and release protein at pH <3.5 (HASLAM, 1989, PATRA; SAXENA, 2011).

2.4.3 Tannin binding agents

Tannin binding agents, notable polyethylene glycol (PEG) and polyvinylpyrrolidone (PVPP) have been widely used as research tools to investigate the effects of tannins both *in vivo* and *in vitro* because they are considered to break already formed tannin-protein complexes, as their affinity for tannins is higher than for proteins (MAKKER et al., 1995). Systematic investigations were conducted on the binding efficiency of PVPP (molecular weights: 10,000, 40,000, and 360,000) and PEG (molecular weight: 2000–35,000) in order to identify the most effective tannin-complexing agents (MAKKAR et al., 1995; 2003; BESHARATI; TAGHIZADEH, 2011)

Bueno et al. (2008) studied the increase in gas production (GP) comparing *in vitro* fermentation of substrates in absence and in presence of binding agents (PEG and PVPP) and they found that PEG seemed to be more efficient to bind tannins than PVPP. The authors showed that the complex formed by PEG and tannins was more stable than the one with PVPP
and tannins.

On other hand, Besharati and Taghizadeh (2011) demonstrated the similarity of PEG and PVPP on their *in vitro* effect of increasing GP, metabolizable energy (ME), net energy (NE), organic matter degradability (OMD) and short chain fatty acids (SCFA) when added to grape by products.

Many *in vivo* studies confirmed the improvement in the nitrogen balance and nutrients digestibility by PEG addition. Jetana et al. (2011) investigated the inactivation of leucaena (*Leucaena leucocephala*) CT by alkaline treatment (NaOH solution) or PEG addition to rice straw and leucaena basal diet fed to Brahman cattle and they found that the NaOH treatment improved the fiber digestion but decrease ($P<0.05$) the nitrogen balance, while the PEG addition increased ($P<0.05$) nitrogen and fiber digestion, as well improved ($P<0.05$) the nitrogen balance. Similarly, Abarghuei et al. (2010) confirmed the improvement of sheep crud protein digestibility, ruminal parameters (pH, ammonia, cellulolytic and proteolytic bacteria

population and protozoa number) and microbial protein yield when replacing alfalfa with grape (*Vitis vinifera sp*.) by-product plus PEG (MW 6000) compared to the control dite based on alfalfa hay.

The ability of PEG to bind and alter the biological activity of tannins depends on tannin type, structural differences, molecular weight and degree of polymerisation. Pellikaan et al. (2011) tested the *in vitro* biological activity of green tea and grape seed with lucerne as a control substrate on extent and rate of gas and CH_4 production. The results confirmed that PEG inclusion with tea tannins decreased CH_4 and GP, whereas grape seed with PEG addition resulted in an increase in gas and CH_4 production. The authors suggested these differences lead to the tannin structural differences between tea and grape seed. Tea tannins are mainly comprised of epigallocatechin gallate plus mono-, di- and trimers of (epi) gallocatechins, while grape seedis similar to quebracho tannins and are composed of dimers of polymers of (epi) catechin and profisetinidins.

The tannin structure in the same plant sample can differ according to the plant part. Theodoridou et al. (2011) investigated the *in vitro* effects of the content and structural characteristics of condensed tannins (CT) in the whole plant, leaves and stems of sainfoin (*Onobrychis viciifolia*) incubated with or without PEG addition on the digestive process in the rumen. The incubation of leaves with PEG had a higher effect on *in vitro* fermentation than that of stems due to different CT characteristics in these parts of the plant.

Not only the tannin structure but also the animal species may affect on the ability of PEG to binding tannins. Rogosic et al. (2008) examined the effect of PEG addition (25 g) on daily intake of Mediterranean shrubs by sheep and goats. Sheep receiving PEG ate more (P=0.002) total shrubs than did control, but no PEG effect was found for goats. The authors suggested that, PEG had a greater influence on sheep than goats when only 3 shrubs were offered. The result is may be related to the fact that fewer shrubs with complementary secondary compounds were offered and that goats appear to have a greater ability to consume and detoxify secondary compounds from mediterranean shrubs.

2.4.4 Effect of tannins on ruminal methanogenesis

Tannin-containing forages and tannin extracts have been demonstrated to decrease methane production both *in vivo* and *in vitro* directly through their antimethanogenic activity and indirectly through their antiprotozoal activity and a reduction in fiber digestion, which decreases H_2 production (TAVENDALE et al., 2005; BHATTA et al., 2009). Recently, from

various studies *in vitro* and *in vivo*, Goel and Makkar (2012) reviewed that CT decrease CH_4 production through reduction in fibre digestion (indirect effect), while hydrolysable tannins appear to act more through inhibition of the growth and/or activity of methanogens and/or hydrogen-producing microbes (direct effect).

For example, Tan et al. (2011) investigated a decreasing rate (linear $P<0.01$; quadratic $P<0.01$) of CH_4 (ml/g DM) production with increasing levels of CT, 0 (control), 10, 15, 20, 25 and 30 mg extracted from *Leucaena leucocephala* hybrid Rendang (LLR) which added for 500 mg of oven dried guinea grass (*Panicum maximum*) using *in vitro* GP procedure. Similarly, Sallam et al. (2010) reported a reduction in CH_4 production ($P<0.05$) by 88, 89 and 91 % in leucaena, acacia and eucalyptus samples, respectively in comparison to alfalfa (control) using *in vitro* GP technique. The authors attributed the reduction by the indirect effect of CT action on methanogenesis by reduced H_2 production form OM digestibility, and by direct inhibitory effects on methanogens.

Abdalla et al. (2012) tested various types of tanniniferous tropical browses i.e, *Arachis pintoi* (ARA), *Crotalaria juncea* (CRT), *Cajanus cajan* (GND), *Dolichos lablab* (LAB), *Leucaena leucocephala* (LEU), *Mucuna cinereum* (MCZ), *Mucuna aterrima* (MPR), *Mimosa caesalpiniaefolia* (SAN), and *Tephrosia candida* (TFR) that showed 21, 0.3, 29, 0.5, 26, 34, 20, 105 and 0.3 g/kg DM of CT. Comparing to Tifton-85 hay (*Cynodon sp*; CT=0.2 g/kgDM) using *in vitro* GP system, among the experimental plants, SAN presented the lowest ($P<0.05$) CH_4 per 100 ml fermentation gas produced, whereas TFR presented the highest values ($P<0.05$) and the other test feeds presented similar values. The authors related this reduction to the CT content since SAN had the highest CT compared with the other plants.

Such reduction in CH_4 emission of SAN was confirmed *in vivo* by the same authors (ABDALLA et al., 2012) since Santa Ines sheep reduce their CH_4 emission by 35.5% without adverse effect on the total tract OM digestibility when received 127 g / kg BW/ day of SAN replacing the Tifton hay in a 70:30 forage to concentrate ratio basal diet of Tifton hay (70%), soybean meal (21% and corn grain (9%).

Similarly, Animut et al. (2008) observed a linear reduction in CH_4 emission in goats that were fed different levels of *Kobe lespedeza* and it was attributed to the presence of CT in this plant.

On the other hand, Beauchemin et al. (2007) did not found any effect ($P>0.05$) of CT extract from quebracho trees (*Schinopsis quebracho-colorado;* contain 91% CT) supplemented to 70 % forage diet (DM basis) as 0, 1, and 2% of dietary DM for 28-d periods on methane emission of Angus heifers. The authors suggested that the lack of effect of

quebracho tannin extract supplementation on CH_4 production was consistent with the lack of effect on total tract DM and fiber digestibility, which could be attributed to the level of CT supplementation, below the threshold required to reduce CH_4 in cattle.

2.4.5 Effect of tannins on rumen fermentation, degradability and total tract digestibility

Generally, the mean effect of tannins on rumen fermentation is mainly related to CT which have been suggested to reduce protein, fiber and amino acids degradation due to ability to interact with other macromolecules such as proteins and carbohydrates (TAN et al., 2011; THEODORIDOU et al., 2011). While hydrolysable tannins may have a less damaging effect on protein digestion than CT because tannins-protein complex may be hydrolyzed in the acidic gastric environment and release the bounded proteins (ABARGHUEI et al., 2011).

The effects of CT on ruminal total SCFA concentration and SCFA pattern have been variable among studies depending on the dosage rate and the sources (BENCHAAR et al., 2008a). Beauchemin et al. (2007) reported that despite a lack of effect of quebracho condensed tannins (QCT) supplementation on total-tract digestibility of DM, increasing supplementation levels of QCT (up to 2% of DMI) tended (P=0.08) to decrease ruminal total SCFA concentration, and decreased acetate molar proportion and the acetate: propionate ratio. In contrast, despite a reduction in total-tract digestibility of OM, Carulla et al. (2005) observed no change in total SCFA concentration in sheep supplemented with *Acacia mearnsii* extract, but molar proportion of acetate decreased and that of propionate increased in sheep fed diets supplemented with CT.

Benchaar et al. (2008a) demonstrated that supplementing dairy cows diet with CT from quebracho trees (QCT, containing 70% tannins, 150 g/cow / day) did not affect (P>0.05) the ruminal pH, total volatile fatty acid concentration, and molar proportions of total volatile fatty acid (acetate, propionate, and butyrate) and tended to decrease (P=0.13) ruminal NH_3-N concentration compared with cows fed the control diet. The authors suggested that the dose of QCT in the study was below the threshold required to cause effect on the rumen microbial fermentation.

Tan et al. (2011) tested the effect of different levels of CT extracted from *Leucaena leucocephala* at 0 (control), 10, 15, 20, 25 and 30 mg supplemented to 500 mg of oven dried guinea grass (*Panicum maximum*) using an *in vitro* GP procedure. The results showed that the reduction in DM degradability was only substantial (22 to 37%) at high CT levels of 20 to 30 mg while, at a lower CT level of 15 mg, the reduction was only 7% and there was a decline

(P<0.01) in nitrogen disappearance with increasing levels of CT inclusion. Total SCFA was reduced (P<0.05) at all levels of CT inclusions as well as GP value, but acetic acid production numerically increased (P<0.05) with increasing CT inclusion, while propionate decreased (P<0.05). An increase in acetate production would be due to increased acetogenesis, a CO_2-reducing process to produce acetate, which is also a way to dispose of metabolic H_2 during microbial metabolism.

Singh et al. (2011) observed a reduction (P<0.05) in the digestibility's of DM, OM, CP, EE, NDF, ADF and ammonia concentrations in goats fed with tannin rich Pakar (*Ficus infectoria*) leaves as compared with the control group (no tannin content), while there were no differences (P>0.05) in the rumen pH, the total SCFA concentration, molar proportions of acetate, propionate, butyrate and acetate: propionate ratio between the experimental and control fed goats. These results could be related to the high tannin content of pakar leaves (8.60 % TT and 7.82 % CT) which also induced a shift in the rumen microbial profile since no changes in total bacterial population but total fungi and cellulolytic bacteria were decreased (P<0.05) with increased (P<0.05) the population of tannin tolerant bacteria.

Theodoridou et al. (2011) studied the relationship between the CT chemical structure and the reactivity of leaves, stems and whole plant of sainfoin (*Onobrychis viciifolia*) CT in two different vegetation cycle periods on rumen degradability and total tract digestibility. Sainfoin CT in leaves and stems greatly increased the *in situ* estimate of forage N escaping from the rumen but did not impact the amount of N disappearing in the intestine, which is the main site of amino acid absorption by ruminants. These CT hardly affected OMD, and did not affect N retention, which is beneficial in ruminant nutrition. The nutritive value of sainfoin was higher at the start of flowering, as the plant was less mature and had more N and less fibre. The higher CT effect at the start of flowering appears to be related to the higher biological activity and lower molecular weight of tannins. The biological activity and content of CT in the whole plant decreased as phenological stage increased. Prodelphinidin: procyanidin ratios of leaves varied with vegetation cycle and phenological stage. The molecular size of CT in the whole plant, as indicated by their mean degree of polymerisation was lowest at the start of flowering and coincided with the higher biological activity and content of CT.

2.4.6 Effect of tannins on rumen protozoa

The effect of tannins on rumen protozoa are conflicting, while some studies reported that there are no effect of tannins on protozoa, others studies confirmed the reduction in protozoa count by tannin rich diets. Chaudhary et al. (2011) found no differences (P>0.05) detected in protozoa count of goats fed with pakar (*Ficus infectoria*) leaves (11.2 % DM basis total tannins) supplemented with or without live culture of tannin degrading bacteria (Isolate-6, 105 to 07 cells/ml) when compared to the control diet (no tannin).

On other instances, Benchaar e al. (2008a) investigated the effect of CT from quebracho trees (QCT, containing 70% tannins, 150 g/cow per day) of lactating cows. Total protozoa counts were not changed by the addition of QCT at 0.05 mg/ml but decreased at the concentrations of 0.1 and 0.2 mg/ml. *Entodiniomorph* numbers were only decreased at the highest concentration of tannins, whereas holotrich numbers were reduced at all tannin concentrations. Whereas, Carulla et al. (2005) observed that feeding tannins from *Acacia mearnsii* extract to sheep had no effects on total counts of protozoa and entodiniomorphs but decreased the numbers of holotrich ciliate protozoa. These results from the previous studies suggested that holotrichs may be more sensitive to CT than entodiniomorphs.

Tan et al. (2011) observed a reduction in protozoa when levels of CT, 0 (control), 10, 15, 20, 25 and 30 mg extracted from *Leucaena leucocepha*la hybrid-Rendang (LLR) added to 500 mg of oven dried guinea grass (*Panicum maximum*). Not only CT affect the protozoa but also hydrolysable tannin (HTs); Abarghuei et al. (2011) tested the effect of HTs in oak leaves (*Quercus libani* Oliv.) on sheep ruminal protozoa count with or without PEG addition. The results confirmed the reduction (P<0.05) of total protozoa, *Isotricha, Diplodinium* and *Eudiplodinium* population in sheep fed oak leaves diet in comparison to those fed the control diet (without tannins) and PEG addition had no effect. In a more recent study, Vasta et al. (2010) found unexpected increase (P<0.05) in the lambs rumen total protozoa count when fed a barley-based concentrate supplemented with quebracho tannins (9.57% of dry matter) compared with the control (same concentrate without tannins addition).

Generally it could be concluded that, tannins level, animal species, individual animal differences and sampling methods affected the rumen protozoa population (YANEZ RUIZ et al., 2004; BENCHAAR et al., 2008a; ABARGHUEI et al., 2011).

2.4.7 What are essential oils (EO)?

Essential oils are blends of secondary metabolites obtained from the plant volatile fraction by steam distillation (GERSHENZON; CROTEAU, 1991). The term “essential” derives from “essence,” which means smell or taste, and relates to the property of these substances of providing specific flavors and odors to many plants. They have important ecological functions as chemical messengers between the plant and its environment, and often exhibit antimicrobial activity against a wide range of bacteria, yeasts, and molds (CALSAMIGLIA et al., 2007).

The most important active compounds (Figure 2) are included in two chemical groups: terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoids. These groups originate from different precursors of the primary metabolism and are synthesized through separate metabolic pathways (Figure 3) (CALSAMIGLIA et al., 2007).

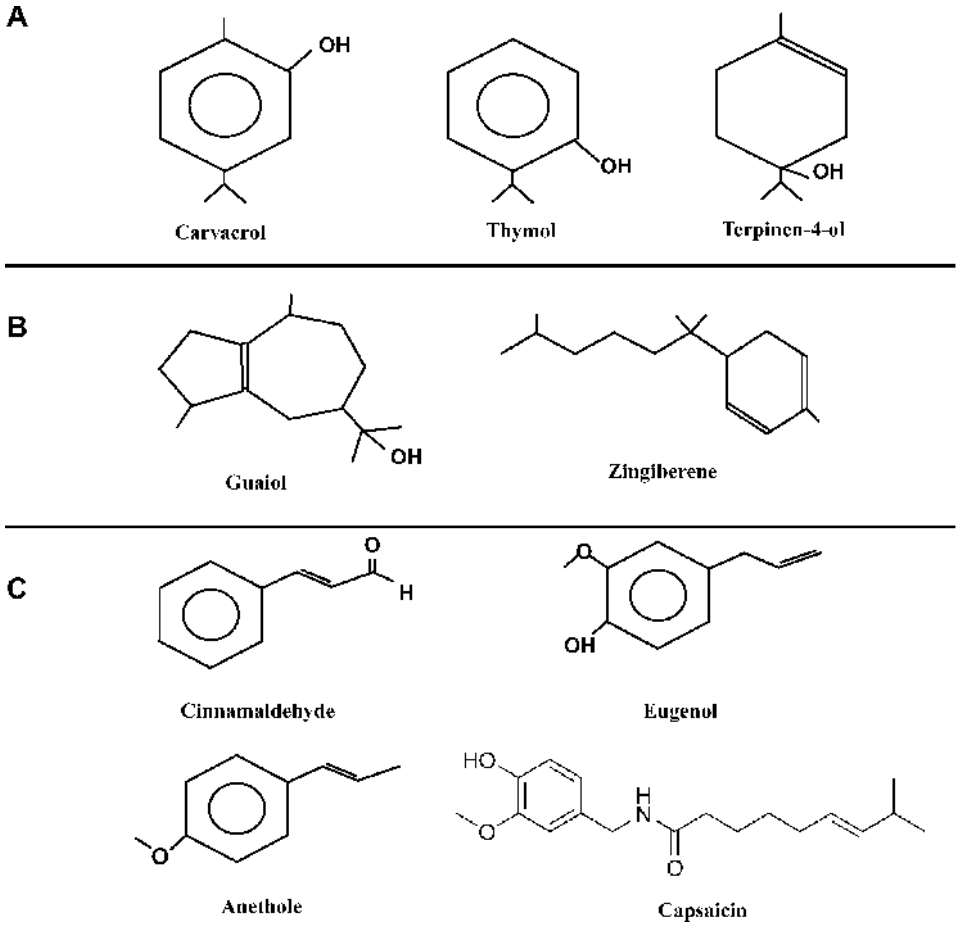

Figure2 - The main monoterpenoid (A), sesquiterpenoid (B), and phenylpropanoid (C) components of essential oils (CALSAMIGLIA et al., 2007)

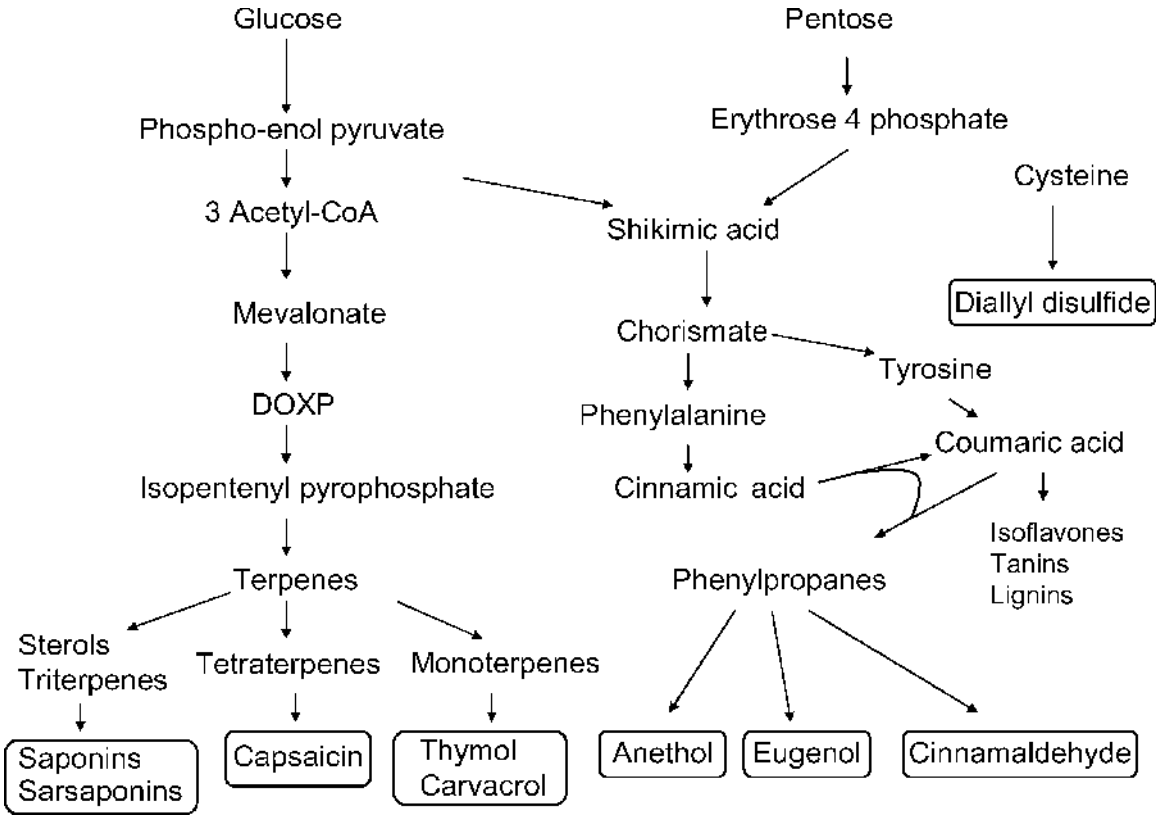

Figure 3 - Metabolic pathways of the biosynthesis of the main plant extract active components (CALSAMIGLIA et al., 2007)

The phenylpropanoids are derived from phenylalanine via the Shikimate pathway and the terpenoids are derived from acetyl-CoA via either the deoxyxylulose (DOXP) or melavonate pathways (EDWARDS; GRATEHOUSE, 1999). Both phenylpropanoids and terpenoids are non-nitrogenous hydrocarbons (ACAMOVIC; BROOKER, 2005).

The phenylpropanoids consist of a C6 benzenic ring with a C3 propionic side chain whereas, the monoterpenes are C10 hydrocarbon dimmers of C5 isoprene units, that may also contain a benzenic ring (DUDAREVA et al., 2004). Lee et al. (2004) reported that there are in excess of 1000 monoterpenes and approximately 50 phenylpropanoids occurring naturally in plants.

Essential oils are found throughout the plant, including roots, the bark, flowers, petals, leaves, fruit bodies and stems (HIRASA; TAKEMASA, 1998). The concentrations of essential oils within plants vary due to stage of growth, plant health (DUDAREVA et al., 2004) and environmental factors such as light, temperature and moisture stress (GERSHENZON et al., 2000).

2.4.8 Effect of essential oils on the ruminal methanogenesis

In the recent years, it has been of immense interest to exploit essential oils and their active components to decrease methanogenesis in the rumen. For instance, Lin et al. (2011) investigated *in vitro* combination of different essential oil active components (i.e. eugenol, carvacrol, citral and cinnamaldehyde) mixed at ratios of 1:2:3:4, 2:1:4:3, 3:4:1:2, 4:3:2:1 and 1:1:1:1 at all the experimental levels of (0, 50, 200 or 500 mg/l culture fluid). The optimal combination for CH_4 production inhibition is when a mixture of these EO at an equal weight ratio is added at 200 mg/l, indicating that a blending of EO components with different action modes together would be a potential way to inhibit ruminal methane.

Agarwal et al. (2009) reported that inclusion of peppermint oil at a concentration of 0.33 ml/l in an *in vitro* GP medium increased methanogen numbers by two fold although there was a decrease in CH_4 production by 20% without affecting SCFA production. In this study, the higher doses (1 and 2 ml/ l) of peppermint oil decreased methanogen populations and CH_4 production. It appears that a decrease in methanogenesis at low doses might be associated with the changes in the rate of methanogenesis by rumen archaea due to the alteration of archaeal community or in the activity of CH_4 producing genes (OHENE; ADJEI et al., 2008).

The differences in antimicrobial activity among essential oils active components additives as well as their doses could affect the CH_4 production. Araujo et al. (2011) tested the high dose of carvacrol, eugenol and 1,8-cineol at 667 mg/L culture fluid and monensin at 2.08 mg/L of buffered rumen fluid using *in vitro* GP technique. The results showed that carvacrol and eugenol reduced ($P \leq 0.05$) CH_4 production by 94.6% and 15.0% compared with control (no additive) while monensin inclusion reduced ($P < 0.05$) CH_4 production by 24.2%. Both of essential oils and monensin decreased the rumen DM degradability while 1,8-cineol did not affect either for CH_4 production or the degradability. Evans and Martin (2000) observed that thymol (400 mg/l), a main component of EO derived from *Thymus* and *Origanum* plants, was a strong inhibitor of CH_4 *in vitro*, but acetate and propionate concentrations also decreased. Sallam et al. (2011) reported that the addition of high doses (75 µl to 75 ml of buffered rumen fluid) of essential oils extracted from *Achillea santolina* and *Artemisia judaica* (the main active component are 16-dimethyl 15-clooactdaiene (60.5%) and piperitone (49.1%), respectively) to 0.5g of 50:50 concentrate: forage inhibited the CH_4 production *in vitro* along with a significant reduction in true degradation of dry matter and organic matter, protozoa count and NH_3-N concentration.

Few studies have evaluated effects of EO and their main components *in vivo* for effects on CH_4 emissions (BENCHAAR et al., 2008b). Mohammed et al. (2004) observed a 19% decrease in CH_4 production in steers that was not accompanied by a reduction in protozoal numbers or feed digestibility using another high supplementation rate (*i.e.*, 20 g/kg of DM intake) of encapsulated horseradish.

Recently, Abdalla et al. (2012) did not found significant differences ($P>0.05$) in the methane emission or diet digestibility of Santa Ines sheep fed on Tifton hay and concentrate diet (in a ratio of 60:40) supplemented with 10 or 20 ml of eucalyptus oil (EuO) for 28 days while Sallam et al. (2009) reported a liner reduction in CH_4 production by 26, 46.8, 77.3 and 85 % when 25, 50, 100 and 150µl of the same EuO in the previous study added to roughage and concentrate diet in a ratio 50:50 without any adverse effect on the dry or organic matter degradability *in vitro*.

These differences between the *in vitro* and *in vivo* studies are compelling evidence that microbial populations may be able to adapt to essential oils over time because *in vitro* studies, in which the adaptation time is short, may discard a product that would work if enough adaptation time had been allowed, but at the same time, a product that is selected based on its short-term effect may not work after a period of adaptation which presents a challenge for commercial application of this feed additive technology (CALSAMIGLIA et al., 2007). But generally it could be concluded that essential oils appear to be natural alternatives to the use of growth-promoter antibiotic additives in animal feeds to reduce CH_4 production.

2.4.9 Effect of essential oils on the rumen fermentation, degradability and total tract digestibility

Essential oils develop their action against bacteria through interacting with the cell membrane (DORMAN; DEANS, 2000). Partly, this activity is due to the hydrophobic nature of the cyclic hydrocarbons, which allows them to interact with cell membranes and accumulate in the lipidic bilayer of bacteria, occupying a space between the chains of fatty acids causing loss of membrane stability (ULTEE et al., 2002). This is not the only mode of action of essential oils. Gustafson and Bowen (1997) reported the potential of essential oils to coagulate some cell constituents, probably by denaturation of proteins like enzymes, nucleic acids.

More addition, the small molecular weight of EO and their active compounds (i.e, carvacrol and eugenol) may allow them to be active in gram-positive and gram-negative bacteria. Unfortunately, this property reduces the selectivity of these compounds against specific populations, making the modulation of rumen microbial fermentation more difficult, and may decrease the fermentation at high concentrations (CALSAMIGLIA et al., 2007).

For instance, recently the combination of different levels of essential oils active components was studied (i.e. eugenol, carvacrol, citral and cinnamaldehyde) by Lin et al. (2011) and concluded that the effects of essential oils active components on rumen fermentation parameters were consistent, irrespective of the component compounds, but a high level of these products would induce the inhibition of rumen fermentation. This hypothesis may partly confirmed previously by Cardozo et al. (2005) who found the lowest concentrations of eugenol, *i.e.* 0.3 and 3 mg/l, tended to increase the ammonia concentration whereas the intermediate level of 30 mg/l had no effect, and the higher levels of 300, 3000 and 5000 mg/l significantly reduced ammonia in an *in vitro* batch culture system. It is evident from the literature that effects of essential oils on rumen fermentation characteristics are largely dependent on the type of diet (or incubation substrate) used, and the type and dose of essential oil, and active components concentrations. Thus Calsamiglia et al. (2007) from an extensive review of the *in vitro, in situ*, and continuous culture-based literature concluded that it is necessary either to report the concentrations of the essential oils active compounds in the plant extracts used in the research, or to use pure products to define activities, doses, and mechanisms of action in an unequivocal form. The authors justify that because the concentration of active components in essential oils can vary widely depending in the cultivar, growing conditions, or processing methods for oil extraction.

Benchaar et al. (2007) examined *in vitro* the individual effects of five EO (cinnamon leaf, clove leaf, sweet orange, oregano, and thyme oils) and four EO active components (carvacrol, cinnamaldehyde, eugenol, and thymol) on rumen fermentation using 51:49 forage: concentrate dairy ration. The results showed that among the EO and their active components investigated only phenolic compounds like carvacrol (400 mg L^{-1}), thymol (200 mg L^{-1}), and eugenol (800 mg L^{-1}) exhibited antimicrobial activity as exemplified by reduced diet fermentability and a shift in SCFA profile from less propionate towards more butyrate. The authors concluded that the types and concentrations of EO and their active components employed to alter ruminal fermentation must be carefully defined before their widespread use in ruminant nutrition can be recommended.

Garcia et al. (2007) observed a similarity in *in vitro* DM, NDF and CP degradability of 250 mg/l carvacrol (C250) and monensin supplement as a dietary additive to rumen fermentors, fed a barley seed: alfalfa hay (70:30) ration. However, they were different in the SCFA profile, since monensin increased the molar proration of propionate and decreased acetate and butyrate but carvacrol decreased the molar proportion of acetate and propionate, but increased butyrate molar proportion.

Essential oils are known also for their inhibition of "hyper ammonia producing bacteria" involved in amino acid deamination (HART et al., 2007). Cardozo et al. (2004), in a continuous culture experiment, were the first to suggest that cinnamon oil (*Cinnamomum cassia*) which contain 75% cinnamaldehyde as the main active component of its composition, modified N metabolism of rumen microorganisms by inhibiting peptidolysis, but the effects on SCFA concentration was negligible.

2.4.10 Effect of essential oils on rumen protozoa

There may be potential to select EO compounds that reduce CH_4 by selectively inhibiting protozoal numbers, which would be expected to decrease CH_4 production because ruminal protozoa provide a habitat for methanogens that live on and within them (BENCHAAR et al., 2008b). However, there are mixed reports on the effects of essential oils on rumen protozoa. The early study of Eadie et al. (1956) showed that certain terpenes and other substances present in plant material had marked toxic properties towards rumen protozoa. However, the effects of EO on some species of protozoa in low concentrations might be stimulatory. For example, addition of a mixture of cinnamaldehyde (180 mg/day) and eugenol (90 mg/ day) to the diets of beef heifers increased the numbers of holotrichs and had no effect on entodiniomorphs, but there was no effect on the numbers of these protozoal species when the mixture contained higher concentrations of cinnamaldehyde (600 mg/day) and eugenol (300 mg/day) (CARDOZO et al., 2006).

Benchaar et al. (2008b) also observed that feeding of 1 g cinnamaldehyde per day to dairy cows increased the number of Isotricha, but had no effect on total protozoa. In contrast, feeding 2 g/day of anise extract containing 100 g/kg of anethol to cattle decreased the numbers of holotrichs and entodiniomorphs (CARDOZO et al., 2006). In contrast, many studies confirmed the reduction of protozoa counts by essential oils supplementation.

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3 TANNINIFEROUS PLANTS AND METHANE EMISSION: RUMINAL METHANE PRODUCTION, DEGRADABILITY AND POST RUMINAL PROTEIN DIGESTIBILITY *IN VITRO*

Abstract

Two experiments were conducted to evaluate four Egyptian tanniniferous plants, *i.e.* leaves of prosopis (*Prosopis juliflora),* acacia *(Acacia saligna),* atriplex *(Atriplex halimus), and* leucaena *(Leucaena leucocephala*) compared with Tifton (*Cynodon sp*) as non tannin hay content for their gas production (GP), methanogenic potential, ruminal fermentation characterstics using semi-automatic system of GP (first experiment) and for rumen and post ruminal protein degradability *in vitro* (second experiment). The results showed that both, acacia and leucaena showed highly condensed tannins (CT) content (63 and 46 eq-g leucocynidin /kg DM, respectively). The ranking of the plants according to their methane inhibition using Tifton as control was acacia > leucaena > atriplex > prosopis (37.9, 35.31, 26.39 and 26.02% based on truly degraded organic matter (TDOM), respectively). Propionate was higher (P=0.002) with corresponding decrease in acetate: propionate ratio (P=0.004) for prosopis and leucaena. Prosopis showed the lowest (P<0.0001) protozoa count, however it had negligible content of tannins. Acacia and Tifton had the lowest (P<0.0001) rumen TDOM compared with the other plants. Both of acacia and leucaena presented lower (P=0.0002) NH_3-N concentration associated with the decreasing (P<0.0001) in rumen protein degradability (RPD), however leucaena showed higher (P<0.0001) intestinal protein digestibility (IPD) than acacia. The current study suggested that the potential methanogenic properties of these experimental browses and rumen degradability are not only related for the tannins content but also for nutrients composition and the highly tannin plants content could be used as supplements to protect the dietary protein from rumen degradation as well as reducing CH_4 production.

Key words: Gas production. Protozoa. Short chain fatty acids. Rumen fermentation.

3.1 Introduction

The productivity of ruminant livestock in the tropics and subtropics areas of developing countries is limited by the poor nutritional conditions which are characterized by highly lignified, low digestible feed from poor and nitrogen (N)-limited native pastures and crop residues, or suffer from a general lack of feed during drought, or even both (ODENYO et al., 1999). Such this sub-standard productivity results not only in greatly increased methane (CH_4) emissions which led to the responsibility of these countries for almost three-quarters of the enteric CH_4 emissions but also a very high cost in terms of CH_4 emissions per unit of product (ALUWONG et al., 2011). And yet, the abatement of ruminal methanogenesis is not of a high priority in these countries and is still highly relevant, because of economic reasons (SOLIVA et al., 2008).

Therefore, the use of browse species containing secondary compounds as feed supplement rich in plant secondary metabolites for ruminants in many parts of the tropics is increasing in order to improve animal performance and reduce CH_4 (GOEL; MAKKAR, 2012). Highly protein tanniniferous plants are apparently promising to overcome the limitations of ruminant production (SOLIVA et al., 2008; ABDALLA et al., 2012).

Although tannins are generally recognized as toxic and anti-nutritional factors but depending on type, concentration and activity of tannins. The moderate levels of condensed tannins (CT) in these plants is related to the protection of dietary protein against rumen degradation by ruminal microorganisms increasing the flux of dietary protein to be absorbed in the intestines resulting in improving amino acid absorption (MIN et al., 2003) for that its intestinal digestion becomes increasingly important.

These plants are not only useful as feed alternatives to the typical low-quality diets but also have potential in terms of methane mitigation (VITTI et al., 2005; BUENO et al., 2008; SALLAM et al., 2010; BHATTA et al., 2012). Various studies have been reported that tannins are able to suppress ruminal methanogenesis directly through their antimethanogenic activity and indirectly through their antiprotozoal activity (BHATTA et al., 2009; GOEL; MAKKAR, 2012). The described anti-protozoal activity of some of these plants is also favorable as it may improve efficiency of microbial protein synthesis due to suppression of the bacteriolytic activity of ruminal protozoa (WALLACE, 2004; HU et al., 2005). This could increase protein flow to the duodenum through protein by-passing the rumen (BROWN; PITMAN, 1991). Thus these plants could be used as dietary supplements targeting both goals, limiting ruminal methanogenesis while improving nitrogen supply.

The objective of this work was to evaluate the potential of tanniniferous browses as anti methanogensis, and to examine rumen degradability and post ruminal protein digestibility *in vitro* of such browses compared with Tifton hay as non-tannin feed.

3.2 Materials and Methods

The chemical analysis and *in vitro* assays were carried out at the Center for Nuclear Energy in Agriculture, University of Sao Paulo (CENA/USP) in Piracicaba, Brazil. All used treatments and techniques were in accordance to the Internal Commission for Environmental Ethics in Experimentation with Animals of CENA/USP. Two experiments were independently performed. The first experiment was designed to study the influence of tanniniferous plants on rumen CH_4 production, degradation and fermentation characteristics using *in vitro* gas production (GP) technique. The second experiment was performed to evaluate the potential of these plants on rumen protein degradability and post rumen protein digestibility *in vitro*.

3.2.1 Browse samples

Egyptian tropical browses such as fodder leaves of prosopis (*Prosopis juliflora*), acacia (*Acasia saligna*), atriplex (*Atriplex halimus*) leucaena (*Leucaena leucocephala*) and Tifton-85 *(Cynodon sp)* hay was used as a control plant. Each plant sample (branches with 5 mm or less of length) were harvested while they were still green and growing from North West coast region of Borg El Arab, Egypt. In general, the climate of this region is arid Mediterranean with scarcity of rain and high radiation. The atmospheric relative humidity ranges from 50 to 75 % and the average annual rainfall is about 100-150 mm, distributed over a period of 15-25 rainy days during autumn and winter, respectively and no rain in summer**.** Plant samples were oven dried at 40^{o}C in air-forced oven for 72 h and ground through a 1 mm screen before travelling to Brazil.

3.2.2 Chemical analysis

All samples were analyzed on dry matter (DM) basis according to AOAC (2006) as for organic matter (OM); crude protein (CP as 6.25×N) and ether extract (EE). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured sequentially using the same sample in filter bags and expressed exclusive of residual ash according to Van Soest (1991) and adapted to Mertens (2002). The NDF were assayed with heat stable amylase. Acid detergent lignin (ADL) was determined by solubilization of cellulose with sulphuric acid (72%) according to Van Soest (1991).

3.2.2.1 Total phenols

The experimental plants (200 mg) were extracted with acetone: water (10 ml; 70:30 v/v) in an ultrasonic bath for 20 min. The contents were centrifuged (4 °C, 10 min, 3,000 × *g*) and the supernatant was kept on ice until analysis. Total phenols were determined with the Folin–Ciocalteau reagent and detected using spectrophotometer at 725 nm (MAKKAR et al., 1993; MAKKAR, 2003). A calibration curve was prepared using increment concentrations as 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10 ml of stock solution of tannic acid (0.1 mg/ml) (Merck GmbH, Darmstadt, Germany). Total phenols were calculated as tannic acid equivalents and expressed as eq-g/kg DM.

3.2.2.2 Total tannins

Total tannins were estimated by precipitating tannins with polyvinyl polypyrrolidone (PVPP, FW111.1, Sigma-Aldrich Inc., St. Louis, USA) following Makkar et al. (1995), 100 mg of PVPP was added in test tube and then 1000 µl of distilled water and 1000 µl of tannins extract (previously discribed) were added.

The mixture was kept after vortex in refrigerator for 15 minutes at 4 °C then centrifuged (Sorvall Superspeed RC2-B, Newton, CT, EUA; 700 g; 10 mints; 4 °C). 1000 µl of supernatant was taken for estimation of non tannin phenol in another test tube and volume of 400 µl with distilled water was added and then processed like that of total

phenol estimation with the Folin–Ciocalteau reagent. Extractable tannins were determined as the difference in total phenolics (measured by Folin–Ciocalteau reagent) before and after treatment with PVPP and were expressed as tannic acid equivalents based on DM.

3.2.2.3 Condensed tannins determinations

Condensed tannins (CT) were measured by the HCl-butanol method, according to Makkar (2003). Half ml of tannins extract was taken in test tube in triplicate and 3.0 ml butanol and ferric ammonium sulphate (0.1 ml) reagents were heated in a boiling water bath for 60 min after covered the tubes with glass marbles. Similarly blank was prepared for each sample but without heating the reagent. After cooling the tubes, absorbance was read at 550 nm and CT was were expressed as leucocyanidin equivalent.

3.2.3 *In vitro* assays

3.2.3.1 Inoculum donors and preparations

Six adult rumen cannulated Santa Inês sheep (60± 2.5 kg of BW) grazing tropical grass pasture and supplemented with ground corn and soybean meal (0.7 kg/100 kg of live weight, 20% crude protein) with free access to a mineral premix and fresh water were used as inoculum donors. Three inoculums were used (two animals/each inoculum) at the same time for evaluating the tested materials. Ruminal liquid and solid fractions were collected separately from each animal before morning feeding and kept in pre-warmed thermo containers (39 ºC) under anaerobic conditions. A liquid fraction was obtained by using a stainless steel probe (2.5 mm screen) attached to a large capacity syringe. Similar volumes (500:500 v/v) of both fractions were blended for 10 s, squeezed through three layers of cheesecloth, and maintained in a water bath (39 ^{o}C) under CO_2 until inoculation took place.

3.2.3.2 *In vitro* gas and methane production

The *in vitro* gas production (GP) assay (THEODOROU et al., 1994) was adapted to a semi-automatic system (BUENO et al., 2005) using a pressure transducer and a data logger (Pressure Press Data 800, LANA, CENA/USP, Piracicaba, Brazil). At the same time of the incubation, three runs using three different inoculums were carried out for each plant sample. In each run, six flasks were prepared for each plant sample: three for determining the truly degraded organic matter (TDOM) and three for measuring the ruminal fermentation parameters and protozoa count. The same system was used for the control (Tifton 85 hay, *Cynodon. spp.*) and for the blank flasks (flasks without substrate containing inoculum + medium) to correct the GP from the inoculum.

A half gram of the air dried ground samples was incubated in serum glass flasks of total volume 160 ml with 50 ml of incubation medium (Menke's buffered medium) and 25 ml of inoculum (then with head space of 85 ml). Flasks were sealed immediately with 20 mm butyl septum stoppers (Bellco Glass Inc, Vineland, NJ, USA), manually mixed, and incubated at 39 ^{o}C in a forced oven (Marconi MA35, Piracicaba, SP, Brazil) for 24 h. Head space gas pressure was measured at 2, 4, 8, 12, and 24 h. Gas production was calculated according to Araujo et al. (2011) by the following equation:

$$V = 7.365 \times p$$

Where: V = gas volume (ml); p = measured pressure (psi)

For CH_4 determination, 2 ml gas were sampled at each time of measuring the pressure, using a 5 ml syringe (Becton Dickson Indústria Cirúrgica LTDA, Curitiba, PR, Brazil) and stored in a 10 ml vacuum tube. After each gas sampling, flasks were vented, mixed and returned to the oven. Methane concentration was determined using a gas chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD, 1.0 mm ID, 1 m length; Ref. no 19809; Restek, Bellefonte, PA, USA). Temperatures of column, injector, and flame ionization detector were 60, 200, and 240ºC, respectively. Helium at 10 ml/min was used as the carrier gas. Concentration were determined by external calibration using an analytical curve (0, 30, 60, 90 and 120; ml/L) prepared with pure CH_4 (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brazil; 995 ml/L purity). Methane production was determined according to Tavendale et al. (2005)

and Longo et al. (2006) as CH_4, ml = (Total gas, ml + Headspace, 85 ml) × CH_4 concentration, ml/ml. Both of GP and CH_4 were expressed based on incubated and degraded organic matter as (ml/g OM and ml/g DOM) and calculated by correcting the values of total gas production and incubated or truly degrade organic matter for the corresponding blank.

3.2.3.3 Rumen degradability and fermentation characteristics

After termination of the incubation at 24h, TDOM was determined in three flasks for each inoculum by inclusion of 70 ml of neutral detergent solution (VAN SOEST et al., 1991) without heat stable α-amylase in each flask and incubated at 105^{o}C for 3 h. The flasks content was filtered in pre-weighed crucibles, washed with hot water then acetone and the residual DM and ash were determined. The partitioning factor (PF) was calculated with the ratio between mg of TDOM and gas volume (ml) at 24 h incubation (BLUMMEL et al., 1997).

The other three flasks content were used for determining ruminal fermentation characteristics. The net release values of NH_3-N were measured according to Preston (1995) using micro-Kjeldahl by steam distillation with sodium tetraborate solution (5%), collected in boric acid solution (20%) and determined by titration with solution of H_2SO_4 (0.05N).

The individual short chain fatty acids (SCFA) were determined by gas-liquid chromatography according to Palmquist; Conrad (1971) but with some modifications. Rumen fluid was centrifuged (15.000 g for 10 mints at 4 °C) to remove large feed particles. 1.6 ml of the supernatant was centrifuged (15.000 g for 15 mints at 4 °C) after adding 0.4 ml solution of meta phosphoric acid (3:1) 25% and formic acid 98-100% plus 0.2 ml of 2-ethyl-butyric acid 100 mM (internal standard, MW = 116.16; Sigma Chemie Gmbh, Steinheim, Germany). After centrifugation, approximately 1.2 ml was transferred to the chromatographic vial. one µl was injected to the gas chromatography [(CG HP 7890A; Injetor HP 7683B, Agilent Technologies, Palo Alto, CA, EUA Column capillary, HP-FFAP (19091F-112; 25 m; 0,320 mm; 0,50 µm; J&W Agilent Technologies Inc.; Palo Alto, CA, EUA)].

The calibration standard curve was prepared by known concentrations of external chromatographic standards (Chem Service, West Chester, PA, EUA) as following, i.e. acetic acid, (99.5%, CAS 64-19-97), propionic (99%, CAS 04/09/79), isobutyric (99%, CAS 79-31-2), butyric (98.7%, CAS 107-92-6), isovaleric (99%, CAS 503-74-2) and valeric (99%, CAS 109-52-4).

Two ml of rumen fluid was mixed with two ml of methylgreen–formalin saline (MFS) solution for microscopic couniting of ruminal protozoa according to the the procedure described by Dehority et al. (1983).

3.2.3.4 Post rumen protein digestibility

A three-step *in situ/in vitro* procedure was used to estimate the rumen protein degradability and the intestinal digestion of proteins according to Calsamiglia and Stern (1995) with some modifications. Four clean and dry Dacron bags were used for each experimental sample containing 3 g of substrate. Dacron bags were incubated *in vitro* in an incubator (TECNAL *in vitro* incubador, model TE-150) for 16 h using three different inoculum (12 replicate /plant) obtained from six rumen cannulated animals used in the GP experiment. The inoculum collection and preparation of incubation medium were done following the same previously procedure of the GP experiment.

The *in vitro* incubator had 4 units, each unit act as separated inoculum and contained 1300 ml medium and 650 ml rumen inoculum. At the end of the incubation time, all the bags were washed for one hour using cold tap water and 2 h in washing machine. The bags were then kept in the freezer for 48 h and rewashed again in washing machine for 2 h and dried at 50 ºC for 48 h. The residue in the bags was used to analyse the N content, which refer to the rumen undegradable protein (RUP).

The residue after ruminal exposure (0.1 g) was incubated for 1 h with pepsin (Sigma, Chemie Gmbh, Steinheim, Germany) HCl (pH=1.9) solution followed by pancreatin (Sigma, Chemie Gmbh, Steinheim, Germany) solution (pH=7.8) for 24 h at 38°C. After incubation, three ml of trichloro actetic acid (TCA, 100%) solution were added to each tube and vortexed.

The tubes were left for 15 min and centrifuged at 10,000×g for 15 min. Five ml of the supernatant was collected for N content determination as an indicator for intestinal protein digestibility – (IPD) by the Kjeldahl method.

3.2.4 Statistical analysis

Comparison between the experimental plants was analyzed in a random regression design and means were compared using the Duncan test. Analysis of variance was carried out using the general linear model procedure (PROC GLM) of SAS (2002) (SAS v 9.1; SAS Inst. Inc., Cary, NC).

3.3 Results

3.3.1 Chemical analysis of browses

The nutrients composition and tannins content of experimental plants are shown in Table 3.1. The lowest OM content was found in atriplex leaves (838 g/kg DM). For CP content, leucaena leaves showed the highest value (268 g/kg DM) while Tifton hay showed the lowest value (74 g/kg DM). The highest content of NDF and ADF were found for Tifton hay (803 and 461 g/kg DM respectively), whereas acacia leaves presented the highest lignin content (163 g/kg DM).

For the tannin content, the results revealed that there were wide variations in the tannins content of the investigated browses, in particular TP and TT, which ranged from 6 to 103 eq-g tannic acid/kg DM and 3 to 88 eq-g tannic acid/kg DM respectively. Leucaena and acacia leaves showed the highest content of TP and TT (103, 91 and 88, 71 eq-g tannic acid/kg DM) respectively. Also, acacia and leucaena leaves were rich in CT content (63 and 46 eq-g leucocynidin/ kg DM, respectively); while the other investigated plants had negligible content of TP, TT and CT.

Table 3.1 - Nutrient composition (g/kg DM) and tannin content of the experimental plants

	Experimental plant				
Item	Tifton hay	Acacia	Atriplex	Prosopis	Leucaena
OM	898	905	838	921	931
CP	74	138	162	179	268
NDF	803	465	372	494	336
ADF	461	428	223	384	228
ADL	65	163	144	144	60
TP	6	91	8	29	103
TT	0	71	4	18	88
CT	0	63	0.2	0.4	46

OM= organic matter, CP=crude protein, NDF neutral detergent fiber, ADF =acid detergent fiber, ADL= lignin, TP=Total phenols (eq-g tannic acid/kg DM), TT=Total tannins (eq-g tannic acid/kg DM), CT=Condensed tannins (eq-g leucocyanidin/kg DM).

3.3.2 Gas and methane production

The *in vitro* ruminal GP and CH_4 production at 24h are presented in Table 3.2. The results showed that the values of GP determined for the incubated organic matter (ml/g OM) were highest (P<0.0001) for atriplex and lowest for acacia, while all the tanniniferous browses showed a similar decrease (P<0.0001) in GP expressed for the truly degraded organic matter (ml/g DOM) compared with the Tifton hay.

When ranking the plants according to their methanogenic properties, the high inhibition observed for acacia and leucaena (37.9 and 35.31 % based on TDOM respectively) whereas both atriplex and prosopis were similar in the CH_4 reduction (26 % based on TDOM respectively) compared with the Tifton as a control.

Table 3.2 - *In vitro* ruminal gas production (GP) and methane production (CH_4) of the experimental plants

	Experimental plants						
Item	Tifton hay	Acacia	Atriplex	Prosopis	Leucaena	SEM	*P*-value
GP (ml/g OM)	150^{bc}	125^{c}	191^{a}	152^{b}	131^{bc}	18.00	<0.0001
GP (ml/g DOM)	376^{a}	297^{b}	250^{b}	236^{b}	266^{b}	39.70	<0.0001
CH_4 (ml/g OM)	13.3^{a}	8.2^{b}	14.7^{a}	11.7^{ab}	8.5^{b}	2.68	<0.0001
CH_4 (ml/g DOM)	26.9^{a}	16.7^{b}	19.8^{ab}	19.9^{ab}	17.4^{b}	5.77	0.01

^{a,b,c} Means within a row without a common superscript letter differ significantly,
SEM=Standard error of means.

3.3.3 Ruminal fermentation characteristics

Ruminal pH, NH_3-N concentration, protozoa account, molar proportions of SCFA are shown in Table 3.3. In the current study, the experimental plants had no significant differences of pH but ruminal NH_3-N concentrations were higher (P=0.0002) for atriplex and prosopis compared with the other plants. All the experimental browses had lower (P<0.0001) total counts of protozoa compared with the control, whereas prosopis showed the least number of protozoa followed by leucaena (2.21 and 2.56 10^5/ml), respectively.

Molar proportion of acetate was not significantly different among all the experimental plants, while propionate was higher (P=0.002) with corresponding decreased in acetate: propionate ratio (P=0.004) with prosopis and leucaena. Among all the experimental browses, leucaena tended to decrease (P=0.03) the butyrate concentration whereas there were no significant differences among all plants in the total SCFA.

Table 3.3 In *vitro* rumen microbial fermentation parameters of the experimental plants

	Experimental plants						
Item	Tifton hay	Acacia	Atriplex	Prosopis	Leucaena	SEM	*P*-value
pH	6.93	6.95	6.94	6.98	6.87	0.131	0.46
NH_3-N (mg/100ml)	24.7^{b}	24.5^{b}	27.4^{a}	30.9^{a}	26.4^{b}	2.580	0.0002
Protozoa (10^5/ml)	3.33^{a}	3.04^{b}	3.00^{b}	2.21^{d}	2.56^{c}	0.134	<0.0001
SCFA (mM)							
C2	45.24	45.57	44.22	46.38	41.98	4.200	0.17
C3	11.28^{b}	10.80^{b}	10.73^{b}	12.22^{a}	11.43^{ab}	0.645	0.002
C4	8.54^{a}	6.87^{ab}	7.09^{ab}	6.65^{ab}	6.50^{b}	1.360	0.03
C5	1.13^{a}	0.86^{b}	0.91^{ab}	0.88^{ab}	0.89^{ab}	0.180	0.03
IC4	1.011^{a}	0.75^{b}	0.85^{ab}	0.84^{ab}	0.79^{b}	0.130	0.01
IC5	1.76^{a}	1.32^{b}	1.64^{ab}	1.31^{b}	1.38^{ab}	0.301	0.01
Total	68.97	65.96	65.72	68.37	62.93	5.072	0.11
C2:C3	4.01^{abc}	4.21^{ab}	4.30^{a}	3.79^{bc}	3.70^{c}	0.301	0.004

[a,b,c] Means within a row without a common superscript letter differ significantly
SCFA= short chain fatty acids, C2= acetate, C3=propionate, C4= butyrate, C5=valeriat, IC_4= iso butyrate, IC5= iso valeriat C2:C3, acetate, propionate ratio,
SEM = standard error of means.

3.3.4 Rumen degradability and post ruminal protein digestibility

Table 3.4 contains the partitioning factor (PF), truly degraded organic matter (TDOM) and post ruminal protein digestibility of the experimental plants. The results showed that both of the Tifton hay and prosopis leaves tended to decrease (P=0.002) the PF compared with the other browses. Acacia and Tifton hay had the lowest (P<0.0001) TDOM compared with the other plants.

The results of the current study confirmed a similarity decrease (P<0.0001) of rumen degradable protein (RDP) between acacia and leucaena leaves while acacia and Tifton hay presented the lowest (P<0.0001) mean values of intestinal protein digestibility (IPD) when compared to other browses. The ranking order of the browse species on the basis of their potential IPD were atriplex ≥ prosopis > leucaena ≥ control ≥ acacia.

Table 3.4- partitioning factor (PF), truly degraded organic matter (TDOM), ruminal protein degradability and post rumen protein digestibility (g/ kg DM) of the investigated plants

	Experimental plants						
Item	Tifton hay	Acacia	Atriplex	Prosopis	Leucaena	SEM	*P*-value
PF	2.42^{b}	3.78^{a}	3.69^{a}	3.08^{ab}	4.12^{a}	0.840	0.002
TDOM	403^{d}	424^{d}	767^{a}	641^{b}	504^{c}	54.2	<0.0001
Protein degradation							
RDP	614^{a}	81^{c}	631^{a}	519^{b}	117^{c}	5.18	<0.0001
RUP	386^{c}	919^{a}	369^{c}	481^{b}	882^{a}	5.18	<0.0001
IPD	416^{cd}	339^{d}	628^{a}	535b^{a}	464^{bc}	6.78	<0.0001

^{a,b,c,d} Means within a row without a common superscript letter differ significantly,
RDP= rumen degradable protein, RUP= rumen undegradable protein, IPD= intestinal protein digestibility.

3.4 Discussion

The results revealed that there are wide variations in the chemical composition of the investigated browses. The CP contents of the browses studied had similar ranges as those stated previously (VITTI et al., 2005; SALLAM et al., 2010; ABDALLA et al., 2012). These current results and those of Sallam et al. (2010) suggested that tanniniferous plants seem to be a good source of protein to improve productivity of ruminant livestock in tropical regions.

For CT content, Sallam et al. (2010) found similar results for the Egyptian acacia and leucaena (61.4 and 32.5 eq-g leucocyanidin kg-1DM), respectively. Prosopis had negligible

CT content, while Bhatta et al. (2002) reported that prosopis leaves contained 7 - 11% tannins. Variations in the analytical method can lead to large variations in the final tannin results (STEWART et al., 2000; MAKKAR, 2003). Acacia and leucaena leaves had CT values as high as 30 – 40 g kg^{-1} DM, which might have both adverse and beneficial effects (BARRY et al., 1986).

There was a wide range within the *in vitro* GP values per unit of incubated OM and degraded OM. Atriplex showed the highest value for GP per unit of incubated OM compared with the other browses but when expressed per unit for degraded OM, Tifton hay had highest value, which may be due to differences in the rumen degradability. García-González et al. (2008) observed that GP and CH_4 emissions are closely related to the amount of rumen fermented OM or the amount of digestible OM thus it is better expressed both of GP and CH_4 per unit degraded OM than per incubated OM.

The highly CH_4 reduction for acacia and leucaena was associated with the high TT and CT content. However prosopis and atriplex had negligible content of TT and CT but they inhibited CH_4 by 26 % (based on TDOM) compared to the Tifton. These results suggested that the potential methanogenic properties of theses experimental browses may not be only related with the tannin content but also for the tannin activity and perhaps the nutrient composition since Tifton had the highest NDF content and highest CH_4 production compared with all the experimental browses.

Low concentration of NH_3-N in acacia and leucaena could be attributed to inhibition of the rumen protein degradability and deamination process by CT. In general, this reduction in ammonia N concentration *in vitro* is accompanied by a reduction in protein degradation. Carulla et al. (2005) also reported that feeding *Acacia mearnsii* extract at 4.1% of DMI (providing 2.5% of tannins on a DM basis) to sheep decreased rumen ammonia concentration and apparent total-tract digestibility of CP.

In general, the anti protozoal activity of tannins was often found to be associated with lower methanogenesis (BHATTA et al., 2009) because protozoa contribute with hydrogen for the reduction of CO_2 to CH_4 by the methanogens (JOUANY; LASSALAS, 1997). However, both of atriplex and prosopis showed similar inhibition of CH_4 (based on TDOM) but not for the protozoa count or for TDOM. In this sense, such relationship between protozoa count and CH_4 was not found for prosopis and it seems that not only tannin content of prosopis may have anti-protozoal factor which cause the reduction of protozoa count. These results also confirmed the role of the protozoa for the OMD especially for fiber degradability.

Bhatta et al. (2012) confirmed that the effects of tannin on protozoal numbers are variable, probably because some tannins have direct effect on methanogenic archaea, which are not associated with the protozoa. While Goel and Makkar (2012) suggested that on inhibition of protozoa, the species belonging to Methanobacteriaceae (living in association with protozoa) declined with an increase in the number of free-living Methanobacteriales. The reduced rate of association of protozoa and methanogens could result in higher interspecies hydrogen transfer between increased population of both hydrogen-producing bacteria (*R. flavefaciens* and *F. succinogenes*) and free-living Methanobacteriales indicating no effect on CH_4 production. Thus our results and those of Bhatta et al. (2012) and Goel and Makkar (2012) indicate that the uni-directional relationship between protozoal numbers and methanogenesis, as affected by tannins, is not obligatory.

The corresponding decreases in acetate: propionate ratio with increasing propionate proportion found for prosopis and leucaena without affecting on the total SCFA or pH confirmed that their CH_4 production reduction was a result of the direction of hydrogen from CH_4 to propionate and not as a general fermentation inhibition (DEMEYER; VAN NEVEL, 1975). These results suggested that the fermentation responses patterns in both of prosopis and leucaena were similar to CH_4 reduction by ionophores (GOODRICH et al., 1984).

Generally, CT may affect the rumen OM degradability through interfering with microbial attachment to feed particles and thus showing significant detrimental effects on the microbial by population inhibiting ruminal fermentation to some extent (BARRY; MCNABB, 1999).The results of the current study confirmed that not only tannins content affect the rumen degradability but also highly fiber content since Tifton and acacia had similarity decrease in TDOM values.

The PF values of the experimental tropical plants were within the range of other feedstuffs. Blummel et al. (1997) showed that the PF of feedstuffs can theoretically vary from 2.75 to 4.41. The increase in GP with Tifton could result in lower partitioning of nutrients to microbial protein synthesis (MAKKAR et al., 1998), and reduced PF value while the higher PF of the tannin containing plants compared with Tifton may be due to their anti-protozoal effect that consequently improving the microbial protein synthesis.

The association decrease of the rumen NH_3-H concentration and RDP for acacia and leucaena reflect that the high concentration of CT was related to decreasing ruminal CP degradation probably through formation of tannin–protein complexes that were minimally degraded by ruminal microbes (MCSWEENEY et al., 1999). Leucaena showed higher IPD than acacia and this may be related to differences in their CP content and in the affinity for re-binding of proteins to CT in the jejunum and ileum as pH increases (MIN et al., 2003). The reactivity between CT and proteins depends partly on the molecular weight, type of tertiary structure, and amino acid content of proteins (MIN et al., 2003). In a review, Min et al. (2003) noted that at equivalent concentrations of different CT sources had variable effects on degradation of CP due to differences in molecular weight and chemical structure influencing the biological activity of CT.

Prosopis showed decrease in RDP and highly IPD value compared with control, these results confirmed the antiprotozoal activity of prosopis. The described anti-protozoal activity of some of these plants is also favorable in this sense as it may improve efficiency of microbial protein synthesis due to suppression of the bacteriolytic activity of ruminal protozoa (WALLACE, 2004; HU et al., 2005). This could increase protein flow to the duodenum by protein by-passing the rumen (BROWN; PITMAN, 1991).

A decrease in the rumen protein degradability in the rumen is beneficial for ruminants as they increase the supply of dietary nitrogen to the lower intestine for production. However the limitation in rumen-degradable protein may result in inadequate ruminal ammonia concentration and suboptimal microbial growth as well as fiber fermentation thus the combinations of such these highly tannins content plants with highly fermented diets will improve the microbial protein synthesis and protect the dietary protein without adverse effect of microbial growth efficiency and the all animal productivity.

3.5 Conclusions

The current study suggested that not only the tannins content might limit the rumen methane production and degradability but also the chemical composition. The tannins association effect of reducing methane production with reducing protozoa count was not confirmed for all the studied plants.

The conclusion with the current results is that the highly tannins content plants could be used as supplements to highly fermented diet in order to protect the dietary protein from microbial activity as well as reducing CH_4 production and thus in the enrollment of reducing environmental pollutants. The found effects may be promising approach to the goal of an improved nutrition for small ruminants in developing countries. More *in vivo* studies, however, are necessary to evaluate the presence of synergistic effects in complete diets when combinations of these plants are used.

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4 CARVACROL AND EUGENOL AS MODIFIERS OF RUMEN MICROBIAL FERMENTATION, AND METHANE PRODUCTION *IN VITRO*

Abstract

Carvacrol (CAR) and eugenol (EUG) were used as natural alternatives modifiers of rumen microbial fermentation and methane (CH_4) production *in vitro* using semi-automatic system of gas production for 24 h incubation. The substrates, 500 mg of Tifton hay plus concentrate (50:50 w/w) were incubated with rumen inoculum (25 ml) and 50 ml buffer with no additives (control) or the treatments monensin (MON) (3 µM/75 ml of culture fluid) as positive control, CAR and EUG at [(5, 10 and 20 µl) and (10, 20 and 30 µl) /75 ml of culture fluid)], respectively. The results indicated that both CAR and EUG at 10 and 20 µl/75 ml of culture fluid respectively presented similarity in rumen total gas production (GP), CH_4 and the truly degraded organic matter (TDOM) when compared to MON. Monensin showed the highest ($P< 0.0001$) molar proportion of propionate and the lowest ($P< 0.0001$) acetate: propionate ratio compared with all the treatments whereas all the tested essential oils bioactive components increased ($P<0.0001$) the molar proportion of butyrate. Although the highest dose of CAR (20 µl) presented the lowest ($P< 0.0001$) CH_4 production, this reduction was associated with reduction of acetate, propionate, butyrate and total short chain fatty acids (SCFA) production, which reflected general fermentation inhibition. All treatments decreased ($P< 0.0001$) protozoa count compared with the control and there were found no significant differences ($P>0.05$) in pH and ammonia (NH_3) concentration. These results suggested that both of CAR and EUG in moderate levels could be also used as natural modifiers of rumen fermentation to decrease CH_4 emission, but in a different mode of action than monensin.

Key words: Gas production. Essential oils. Monensin. Short chain fatty acids.

4.1 Introduction

Rumen fermentation process has energy losses (methane; CH_4) and protein losses (ammonia N) inefficiencies that may limit production performance and contribute to the release of greenhouse gases to the environment (BUSQUET et al., 2006). Ionophores like monensin (MON) have been very successful in reducing CH_4 and protein losses in the rumen but the use of antibiotics in animal feeds is facing reduced social acceptance because of the appearance of residues and resistant strains of bacteria that may have effects on human health, so their use has been banned in the European Union (RUSSELL; HOULIHAN, 2003). Therefore, it is necessary to investigate the reduction of CH_4 emission from ruminants using natural feed resources that could have the same effect as ionophores without adverse effect on the rumen degradability (PATRA, 2011).

Essential oils are plant secondary metabolites responsible for the odor and color of plants and spices, and their antibacterial, antifungal, and antioxidant properties make them useful as natural additives in animal feeds. During the last few decades, number of studies have been evaluated the ability of essential oils to reduce enteric CH_4 production that selectively inhibit rumen methanogenesis without depressing feed digestion (BENCHAAR et al., 2007; BENCHAAR; GREATHEAD, 2011)**.** Phenolic components such as eugenol [(4-allyl-2-methoxyphenol; C10H12O2) present in clove bud)], or carvacrol [(2-methyl-5-(1-methylethyl) phenol; $C_6H_3CH_3(OH)(C_3H7)$ present in oregano)], are responsible for the antibacterial properties of many essential oils (DORMAN; DEANS, 2000). However, there is little information on their dose response effects on rumen CH_4 production (MACHEBOEUF et al., 2008; SOLTAN et al., 2010).

This study was conducted to assess the potential benefits of the most phenolic active components for the essential oils (CAR and EUG) as natural modifiers of rumen fermentation to decrease CH_4 emission in sheep diets *in vitro*.

4.2 Material and Methods

The chemical analysis and *in vitro* assays were done at Center for Nuclear Energy in Agriculture, University of Sao Paulo (CENA/USP), Piracicaba, Brazil. All used treatments and techniques were in accordance to the Internal Commission for Environmental Ethics in Experimentation with Animals of CENA/USP.

4.2.1 Chemical composition and treatments

Feed and chemical compositions of the substrate control basal diet are shown in (Table 4.1). The feed sample was chemically analyzed on dry matter (DM) basis according to AOAC (2006) as for organic matter (OM); crude protein (CP as 6.25×N) and ether extract (EE). The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were measured according to Mertens (2002). The ADF and NDF were assayed with a heat stable amylase and expressed exclusive of residual ash. The ADF was obtained by sequential extraction of NDF residue and ADL was determined by solubilization of cellulose with sulphuric acid exclusive residual ash (VAN SOEST, 1991).

Table 4.1 - Ingredients and chemical composition of the incubated substrate *in vitro* (total mixed ration of diet 50:50%)

Composition	%
Ingredients (%)	
Tifton hay	50
Ground corn	32.7
Soybean meal	15.0
Limestone	1.0
Mineral premix	1.3
Chemical composition (% on DM basis)	
Dry matter (DM)	89.9
Organic matter (OM)	92.3
Crude protein (CP)	15.2
Ether extract (EE)	1.9
Neutral detergent fiber (NDF)	54.8
Acid detergent fiber (ADF)	25.6
Acid detergent lignin (ADL)	4.2

The treatments were consisted of the substrate diet with no additives (control) or pure carvacrol (CAR) and eugenol (EUG) (as 100% CAR or EUG from GRASP Ind. e Com. Ltda (Curitiba, PR, Brazil) at (5, 10 and 20 µl) and (10, 20 and 30 µl) /75 ml of culture fluid), respectively and Monensin (MON), M5273; Sigma Aldrich Co., St. Louis, MO; MW =

692.85)) was add at 3 µM/75 ml of culture fluid as positive control by preparing as stock solution by diluting 15.6 mg in 1.0 ml of pure ethanol. A stock solution (10 µl) was added to each flask to achieve a final MON concentration of 2.08 mg/l of buffered rumen fluid because at this concentration, MON could decrease CH_4 without adverse effect on the OM degradability and these amount of ethanol have not effects on the fermentation process *in vitro* according to Araujo et al. (2011) so ethanol was not included for all other flasks.

4.2.2 Inoculum donors and preparations

Six adult rumen cannulated Santa Inês sheep (60 ± 2.5 kg BW) grazing tropical grass pasture and supplemented with ground corn and soybean meal (0.7 kg/100 kg of live weight, 20% crude protein) with free access to mineral premix and fresh water were used as inoculum donors (two animals / each inoculum). Ruminal liquid and solid fractions were collected according to Bueno et al. (2005) from each animal before morning feeding and kept in pre-warmed thermo containers (39ºC) under anaerobic conditions. Similar volumes (500:500 v/v) of both fractions were blended for 10 s, squeezed through three layers of cheesecloth, and maintained in a water bath (39^{o}C) with flushing of CO_2 until inoculation took place.

4.2.3 *In vitro* gas and methane production

The *in vitro* gas production (GP) assay (THEODOROU et al., 1994) was adapted to a semi-automatic system (BUENO et al., 2005) using a pressure transducer and a data logger (Pressure Press Data 800, LANA, CENA–USP, Piracicaba, Brazil). At the same time of the incubation, three runs using three different inoculums (2 sheep / inoculum) were carried out for each treatment. In each run, four flasks were prepared for each treatment: two for determining the truly degraded organic matter (TDOM) and two flasks for measuring ruminal fermentation parameters and protozoa count. For each inoculum, an internal standard (Tifton 85, *Cynodon*. *spp.*) hay was included to enable adjustments among runs and for blanks (flasks without substrate containing inoculum + medium) were used for each inoculum to measure the fraction of total GP due to substrate in inoculm, and these values were used to calculate net GP.

Half gram of the substrate with or without additives was incubated in glass flasks of total volume 160 ml and head space 85 ml with 50 mlof incubation medium (Menke's buffered medium) and 25 mlof inoculum. Flasks were sealed immediately with 20 mm butyl

septum stoppers (Bellco Glass Inc, Vineland, NJ, USA), manually mixed, and incubated at 39°C in a forced air oven (Marconi MA35, Piracicaba, SP, Brazil) for 24 h. Head space gas pressure was measured at 2, 4, 8, 12, and 24 h. Gas production was calculated according to Araujo et al. (2011) by the following equation:

$$V = 7.365 \times p$$

Where: V = gas volume (ml); p = measured pressure (psi)

For CH_4 determination, 2 ml gas were sampled at each preasure measuring time using a 5 ml syringe (Becton Dickson Indústria Cirúrgica LTDA, Curitiba, PR, Brazil) and stored in a 10 ml vacuum tube. After each gas sampling, flasks were vented, mixed and returned to the oven.

Methane concentration was determined using a gas chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD, 1.0 mm ID, 1 m length; Ref. n° 19809; Restek, Bellefonte, PA, USA). Temperatures of column, injector, and flame ionization detector were 60, 200, and 240ºC, respectively. Helium at 10 ml/min was used as the carrier gas. Methane concentration was determined by external calibration using an analytical curve (0, 30, 60, 90 and 120; ml/L) prepared with pure CH_4 (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brazil; 995 ml/L purity) and total methane production was calculated according to Tavendale et al. (2005) and Longo et al. (2006) as follows:

CH_4, ml = (Total gas, ml + Headspace, 85 ml) × CH_4 concentration, ml/ml

Gas production was expressed as ml/g OM and CH_4 for truly degraded organic matter as ml/g TDOM and as % from net GP by correcting the values of total gas production, CH_4, incubated and truly degrade organic matter for the corresponding blank.

4.2.4 Rumen degradability and fermentation characteristics

After termination of the incubation at 24h, TDOM was determined by inclusion of 70 ml of neutral detergent solution (VAN SOEST et al., 1991) without heat stable α-amylase in each flask and incubated at 105°C for 3 h. The flasks content were filtered in pre-weighed

crucibles, washed with hot water then acetone and the residual of DM and ash were determined. The partitioning factor (PF) was calculated as a ratio between mg of TDOM and total gas volume (ml) at 24 h incubation (BLUMMEL et al., 1997).

The other two flasks content were used for determining fermentation characteristics, The SCFA were determined by gas-liquid chromatography following the method of Palmquist; Conrad (1971) but with some modifications. Rumen fluid was centrifuged (15.000 g for 10 mints at 4 °C) to remove large feed particles. 1.6 ml of the supernatant was centrifuged (15.000 g for 15 mints at 4 °C) after adding 0.4 ml solution of meta phosphoric acid (3:1) 25% and formic acid 98-100% plus 0.2 ml of 2-ethyl-butyric acid 100 mM (internal standard, MW = 116.16; Sigma Chemie Gmbh, Steinheim, Germany). After centrifugation, approximately 1.2 ml was transferred to the chromatographic vial. One µl was injected to the gas chromatography [(CG HP 7890A; Injetor HP 7683B, Agilent Technologies, Palo Alto, CA, EUA Column capillary, HP-FFAP (19091F-112; 25 m; 0,320 mm; 0,50 µm; J&W Agilent Technologies Inc.; Palo Alto, CA, EUA)].

The calibration standard curve was prepared by known concentrations of external chromatographic standards (Chem Service, West Chester, PA, EUA) as following, acetic acid, (99.5%, CAS 64-19-97), propionic (99%, CAS 04/09/79), isobutyric (99%, CAS 79-31-2), butyric (98.7%, CAS 107-92-6), isovaleric (99%, CAS 503-74-2) and valeric (99%, CAS 109-52-4).

Two ml of rumen fluid was mixed with two ml of methylgreen–formalin saline (MFS) solution for micrscopic protozoa count according to the procedure described by Dehority et al. (1983). Total NH_3-N contents were measured in the rumen fluid supernatant according to Preston (1995) using micro-Kjeldahl by steam distillation with sodium tetraborate solution (5%), collected in boric acid solution (20%) and determined by titration with 0.05N H_2SO_4

4.2.5 Statistical analysis

Data were subjected to analysis of variance (ANOVA), using the General Linear Model procedure (GLM) of SAS software package (2002). The used model was: $Y= \mu + Fi + e$, where µ is overall mean, *Fi* is the treatment effect, *e* is error term. Experimental units were runs and replicates in the same run considered as repetitions. The significant differences between individual means were identified by using Tukey test.

4.3 Results and Discussion

4.3.1 Gas and methane production

The effect of the essential oils bioactive components supplementation on the rumen GP and CH_4 production are presented in Table 4.2. The results showed that, the control basal diet had the highest (P<0.0001) values of GP, while there were no significant (P>0.05) differences detected among MON and all the essential oils bioactive components treatments. Monensin, EUG 30µl and CAR 20µl were decreased (P=0.0006) CH_4 production compared with the control basal diet.

When ranking the treatments according to their methanogenic properties compared with the control, the highly CH_4 reduction potential observed for CAR 20µl, while MON, EUG 30 µl and CAR 10 µl were similar in the CH_4 reduction. However, the methanogenic population was not measured but these antimethanogenic effects for both CAR and EUG could partly explained by their antimicrobial activity due to the presence of a hydroxyl group in the phenolic structure (ULTEE et al., 2002).

Compounds with phenolic structures have a broad spectrum of activity against a variety of both Gram-positive and Gram-negative bacteria (HELANDER et al., 1998). The mechanism of action by which phenolic compounds are thought to exert their antimicrobial activity is through the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow active transport, coagulation of cell contents, reduction in the synthesis of ATP and finally to cell death (ULTEE et al., 2002).

4.3.2 Rumen degradability and fermentation characteristics

Table 4.3 presents the TDOM, PF, pH, NH_3-N and protozoa count of the experimental essential oils bioactive components compared with the control basal diet.

The current study confirmed the higher antimicrobial activity for CAR compared with MON or EUG, since the highest dose of the CAR (20 µl) present the lowest GP (P=0.0001), CH_4 (P=0.0006), TDDM (P=0.0014) and TDOM (P=0.0005), and had the highest PF value (P=0.0004) compared with the all other treatments. However there was a similar effect observed amoung MON, EUG 20 µl and CAR 10 µl either for the degradability or PF (Table 4.3).

Calsamiglia et al. (2006) reported that the lipophilic properties for both MON and phenolic essential oils such as CAR and EUG make their activity stronger against gram positive bacteria than to gram negative bacteria (CIMANGA et al., 2002). However the external membrane of gram-negative bacteria is not completely impermeable to hydrophobic substances, the lower molecular weight molecules of the phenolic essential oils than the MON allow them to interact with water (through hydrogen bridges), cross the cell wall slowly by diffusion through the layer of lipopolysaccharides or through membrane proteins and interact with the lipid bilayer of cells (DORMAN; DEANS, 2000). Therefore it could be attributed the higher antimicrobial activity of CAR due to the differences between CAR and EUG in their molecular weight. In this sense, higher levels of CAR may affect the rumen OM degradability (Table 4.3) throughout the interference with microbial attachment to feed particles and showed significant detrimental effects on the microbial population inhibiting ruminal fermentation to some extent (BARRY; MCNABB, 1999).

Recently, gas and methane reduction by such these types of the essential oils active components was observed by Araujo et al. (2011), when high dose at 667 mg/l buffered rumen fluid of carvacrol, eugenol was incubated with half gram of substrate consisting of 800:200 (w/w) concentrate: forage diet for 16 h that led to a reduction ($P \leq 0.05$) in the total GP (39.7and 97.9 ml /g TDOM for carvacrol and eugenol, respectively versus 115.0 ml for the control basal diet). While, CH_4 production was 0.6 and 3.7 ml/g TDOM for carvacrol and eugenol, respectively versus 11.2 ml /g TDOMfor the control basal diet. Those results and our founding confirmed the higher antibacterial effect of carvacrol compared with eugenol.

Table 4.2 - Effect of different levels of eugenol and carvacrol on ruminal gas and methane (CH_4) production *in vitro*

Diets	GP	CH_4	CH_4 reduction
	(ml/g OM)	(ml/g TDOM)	potential (%)
Control	139 [a]	19.9[a]	-
Monensin	109[abc]	12.5[bc]	37.2
Eugenol 10µl	134 [ab]	17.3[ab]	13.1
Eugenol 20µl	121[ab]	16.0[abc]	19.6
Eugenol 30µl	100[bc]	12.2[bc]	38.7
Carvacrol 5µl	121[ab]	15.9[abc]	20.1
Carvacrol 10µl	102 [bc]	13.0[abc]	34.7
Carvacrol 20µl	83.8[c]	8.67[c]	56.4
SEM	29.901	5.500	-
P value	0.0001	0.0006	-

[a,b,c] Means in a column with different superscripts differ significantly ($P < 0.05$).

Neither MON nor the experimental essential oils active components had significant effect on term of ruminal pH and NH_3-N concentrations. These results suggested that none of the doses examined of the essential oils had no dramatic impact on the deaminase activity of ruminal microorganisms and the reduction in the OM degradation with the highest dose of CAR could be related to reduction in fiber degradability mainly through acting on cellulolytic species, therefore the diet type is very critical when such essential oils will be used as supplement. All the treatments decrease (P<0.0001) the protozoa count compared with the control basal diet (Table 4.3). In general, the anti protozoal activity often found to be associated with a lower methanogenesis because protozoa contribute hydrogen for the reduction of CO_2 to CH_4 by the methanogens (BHATTA et al., 2009). These results also confirmed the role of the protozoa for the organic matter degradability especially for fiber degradability.

Table 4.3 - Effect of different levels of eugenol and carvacrol in rumninal degradability, fermentation parameters and protozoa count *in vitro*

Diets	TDOM (g/kg)	PF	pH	NH_3-N (mg/100ml)	Protozoa (10^5/ml)
Control	686^{a}	4.49^{b}	6.81	24.1	4.36^{a}
Monensin	639^{ab}	5.18^{b}	6.79	27.1	3.28^{bc}
Eugenol 10µl	654^{ab}	4.46^{b}	6.79	25.6	3.96^{ab}
Eugenol 20µl	609^{abc}	4.79^{b}	6.83	28.4	3.16^{c}
Eugenol 30µl	562^{bc}	5.34^{b}	6.84	29.1	3.04^{c}
Carvacrol 5µl	609^{abc}	4.65^{b}	6.80	28.7	3.41^{bc}
Carvacrol 10µl	572^{bc}	5.14^{b}	6.86	28.4	3.00^{c}
Carvacrol 20µl	523^{c}	9.21^{a}	6.87	22.9	2.73^{c}
SEM	38.60	1.123	0.040	4.142	0.5000
P value	0.0005	0.0004	NS	NS	<0.0001

TDOM= truly degraded organic matter, PF= Partitioning factor.
a,b,c Means in a column with different superscripts differ significantly. SEM=Standard error of means, NS=not significant.

Results in Table 4.4 are in agreement with *in vitro* and *in vivo* studies which demonstrated that monensin decrease of the CH_4 production by reducing (P<0.0001) the proportion of acetate and butyrate and acetate to propionate ratio, while increased (P< 0.0001) the proportion of propionate without affecting total SCFA concentration (CASTILLEJOS et al., 2006). All the doses of both of CAR and EUG showed an increase (P<0.0001) in the molar proportion of butyrate compared with MON without effect on the acetate, propionate, total SCFA and branched chain volatile fatty acids (BCVFA) except for the highest dose of CAR (20 µl) which decreased all the individual and total SCFA.

Benchaar et al. (2007) reported that the addition of 400 mg L^{-1} of CAR on *in vitro* batch culture 24 h with a 51:49 forage: concentrate dairy ration [16.7% crude protein (CP), 34.4% neutral detergent fiber (NDF)] strongly decreased propionate molar proportion and increased butyrate. On the other hand, Busquet et al. (2006) found that 300 mg L^{-1} of CAR on *in vitro* batch culture with a 50:50 forage: concentrate diet decreased the proportion of propionate and increased that of butyrate but at 3,000 mg/L, CAR increased the proportion of propionate and decreased the proportion of butyrate without effect on acetate. Such effect of CAR in reducing the propionate concentration was not found in the current study.

Castillejos et al. (2006) found that the addition of EUG at 500 mg/ L in *in vitro* batch culture (24 h) with 60:40 forage: concentrate diet reduced the proportion of propionate and BCVFA without affecting total SCFA concentration, while when use the same dose and diet in the dual-flow continuous culture fermenters (6 days), EUG increased the proportion of butyrate, propionate and decreased the acetate and BCVFA. This variability in the results suggesting that the effect of both of CAR and EUG on the fermentation pattern depending on the dose, diet type, incubation time and also in the *in vitro* technique (i.e., gas production versus batch or continuous culture).

Table 4.4 -Effect of different levels of eugenol and carvacrol in short chain fatty acids *in vitro*

	Short chain fatty acids (mmol)							
Diets	C2	C3	C4	C5	IC4	IC5	C2/C3	Total
Control	47.3^{a}	11.5^{b}	11.1^{ab}	0.87	0.95^{a}	1.72^{a}	4.1^{b}	73.4^{a}
Monensin	48.7^{a}	14.7^{a}	9.3^{bc}	0.84	0.69^{ab}	1.41^{ab}	3.3^{c}	75.7^{a}
Eugenol 10µl	48.5^{a}	11.5^{b}	11.5^{a}	0.90	0.96^{a}	1.77^{a}	4.2^{b}	75.1^{a}
Eugenol 20µl	43.8^{a}	10.5^{b}	11.5^{a}	0.87	0.86^{a}	1.64^{a}	4.2^{b}	69.1^{a}
Eugenol 30µl	40.8^{a}	9.8^{b}	11.6^{a}	0.87	0.79^{ab}	1.55^{ab}	4.2^{b}	65.4^{a}
Carvacrol 5µl	46.3^{a}	11.4^{b}	12.3^{a}	0/91	0.92^{a}	1.75^{a}	4.1^{b}	72.6^{a}
Carvacrol 10µl	45.5^{a}	11.3^{b}	11.9^{a}	0.91	0.87^{a}	1.72^{a}	4.1^{b}	71.6^{a}
Carvacrol 20µl	32.5^{b}	5.5^{c}	8.9^{c}	0.77	0.42^{b}	0.82^{b}	5.9^{a}	49.0^{b}
SEM	5.84	1.54	1.49	0.31	0.42	0.79	0.27	9.33
P value	<0.0001	<0.0001	<0.0001	NS	0.031	0.006	<0.0001	<0.0001

C2, acetic acid; C3, propionic acid; C4, butyric acid, C5=valeriat, IC4= iso butyrate, IC5= iso valeriat C2:C3, acetate, propionate ratio a, b, c Means in a column with different superscripts differ significantly, NS= not significant.

All the observed results of the current study confirmed that the higher antimicrobial activity of CAR since the highest level of CAR (20 µl) decreased the CH_4 production by decreasing OM degradability and caused general fermentation inhibition but the moderate levels of both of CAR and EUG are promising to reduce CH_4 production without adverse effect on the OM degradability. The results showed that this occurred in a different way from

MON by shifting SCFA pattern towards more butyrate without affecting on the total SCFA and through their antiprotozoal effect. These differences between MON and the essential oils could be due to MON could inhibit gram-positive bacteria, which are involved in fermentation processes that produce, among other products, acetate, butyrate, formate, lactate, hydrogen, and ammonia (HELANDER et al., 1998) whereas, both of CAR and EUG inhibit gram positive and gram negative bacteria (RUSSELL; STROBEL, 1989) but it seems that the major butyrate producer, *Butyrivibrio fibrisolvens* was not inhibited either by CAR or EUG addition.

4.4 Conclusions

Monensin and both of CAR and EUG at 10 and 20 µl/75 ml of culture fluid respectively showed similar GP, CH_4 inhibition, TDOM and protozoa count but in different SCFA pattern. Monensin increased the propionate concentration and decreased the acetate: propionate ratio but both of CAR and EUG increased the butyrate concentrations without effect on the total SCFA compared with MON but the highest level of CAR decreased the GP and CH_4 production by inhibiting fermentation activity and decreased the OMD.These results suggesting that the moderate levels of both of CAR and EUG are an effective natural alternative to monensin in sheep diets for reducing CH_4 emission *in vitro*. However more in vivo studies with sufficient adaptation feeding conditions are required.

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5 EFFECT OF DEACTIVATION OF CONDENSED TANNINS OF *LEUCAENA LEUCOCEPHALA* DIET ON APPARENT DIGESTIBILITY, NITROGEN BALANCE, RUMINAL FERMENTATION AND METHANE EMISSION *IN VIVO*

Abstract

This experiment was carried out to assess the effect of tannins content and biological activity of leucaena *(Leucaena leucocephala*) on *in vivo* total tract apparent digestibility, nitrogen balance, ruminal fermentation and methane emission. Six adult rumen cannulated Santa Inês sheep (70 ± 2.5 kg of BW) adapted to the basal diet (70% Tifton hay (*Cynodon. spp.*) + 21% ground corn, 9% soybean meal) were individually divided into three experimental diets completing a double latin square design (3 treatments, 3 periods, 6 animals). For 17 d (10 d adaptation and 7 d data collection period), the animals were fed *ad libitum*, one of the following diets: control (CNTRL - basal diet only), leucaena diet (LEUC -123 and 8.8 g/kg DM of total tannins and condensed tannins respectively), which consisted of the basal diet replaced by 0.50 of Tifton hay with leucaena and the leucaena diet plus 20 g/day/animal of polyethylene glycol (LPEG). Leucaena shade dried was ground before being thoroughly mixed with required quantity of concentrate and Tifton. The LEUC either with or without PEG increased the DM intake (P=0.009) and nitrogen intake (P=0.005) compared with CNTRL. Although apparent total-tract digestibilities of DM, OM, CP, NDF, and EE were not different (P>0.05) among the dietary treatments, LEUC decreased (P= 0.0009) ADF and tended to decrease (P=0.086) the CP digestibility compared with the CNTRL. These reduction were associated with rumen NH_3-N reduction (P<0.0001) compared with LPEG. Leucaena diets (LEUC and LPEG) improved (P=0.012) N retention compared with the CNTR and decreased (P<0.0001) the CH_4 emission per kg digested OM by 14 and 11%, respectively as well as decreased (P<0.0001) the C2/C3 ratio compared with CNTRL. However, LPEG improved (P=0.012) the acetate and butyrate (P=0.0034) compared with the LEUC. These findings highlight not only the nutritional value of leucaena diet but also the potential for mitigating methane emission and urinary nitrogen execration by sheep.

Keywords: Tannin content. Polyethylene glycol. Rumen fermentation. Ammonia concentarion.

5.1 Introduction

Leucaena leucocephala (leucaena) is a tropical tanniniferous legume plant, fast growing, grows actively in the dry season (KEOGHAM, 1980) and has been shown to have great potential to improve metabolic nitrogen supply for ruminants due to its high protein content (leaves contain 25-30% crude protein (YUANGKLANG et al., 2011) as well as condensed tannin (CT) content (61.4 and 32.5 eq-g leucocyanidin kg^{-1}DM in leaves) that promising to limiting the growth of ruminal methanogenesis in sheep (SALLAM et al., 2010).

Feeding CT containing diets to ruminants have been extensively assessed for their antimicrobial effects and their potential to modulate ruminal fermentation (BENCHAAR et al., 2008) for reducing CH_4 emission (CARULLA et al., 2005; TAN et al., 2011) without adverse effect on the total tract nutrients digestibility (PUCHALA et al., 2005; BENCHAAR et al., 2008) and for reducing the execrated urine nitrogen (BEN SALEM et al., 2000; JETANA et al., 2011).

However, in most of those studies, the reduction in CH_4 was confounded with their tannins content and with changes in forage quality (legumes vs. grass), such as lower NDF content and nitrogen content. Thus, there is still considerable uncertainty about the effectiveness of tanniniferous legumes tannin content to reduce enteric methane emissions from cattle (CARULLA et al., 2005; BEAUCHEMIN et al., 2007; RODRÍGUEZ et al., 2010).

For example, Carulla et al. (2005) studied the effect of supplemented *Acacia mearnsii* tannins extract at two levels, 0 and 2.5% of the diet DM to a partial replacement ryegrass diet by legumes and they observed a significant CH_4 reduction per kg of intake by 13% when acacia tannins were supplemented. However, the replacement of grass by legumes demonstrated no advantage in reducing methanogenisis or excreting N in faeces or urine. However, Rodríguez et al. (2010) found that the replacement of tropical grass by tanniniferous legumes plant that have the nutrients-tannin complexes already in the diet reduced the CH_4 *in vitro* either with or without polyethylene glycol (PEG) which probably was related with the better synchronization of nitrogen and energy available for microbial utilization.

We hypothesized in the current study that, the supplementation of tannin binding agent i.g. PEG to partial replacement of Tifton hay based diet by leucaena would favorably release the naturally tannin- nutrient complexes that is already established in the leucaena diet and enhance the intake, nutrients digestibility, nitrogen balance, rumen fermentation by sheep.

5.2 Material and Methods

The chemical analysis and *in vivo* assays were done at Center for Nuclear Energy in Agriculture, University of Sao Paulo (CENA/USP), Piracicaba, SP – Brazil. All used treatments and techniques were in accordance to the Internal Commission for Environmental and Ethics in Experimentation with Animals of CENA/USP.

5.2.1 Leucaena material and treatments

Consumable parts of leucaena (leaves, flowers, pods and small stem) were harvested between 08:00 h and 10:00 h during the dry season along the Piracicaba river shore in Piracicaba, Sao Paulo state, Brazil. Random plant samples (± 2 kg) were separated immediately and dried at 55º C for 48h to assess the propositional leucaena parts as 46.8, 5.3, 35.7 and 13% for leaves, flowers, pods and small stem, respectively while the harvested leucaena was ground through a 0.5cm screen and allowed a further 96h for shade drying before being pooled bagged and stored. Plant samples (± 2 kg) were collected in triplicate from the bags at the beginning and the end of the *in vivo* experiment and ground to pass through a 0.25mm screen for tannin, mimosine and 2,3-dihydroxypyridine analysis.

5.2.2 Animals and experimental design

Six adult rumen cannulated Santa Inês sheep (70 ± 2.5 kg of BW) were adapted for 17 d to a Tifton 85 hay basal control diet *ad libitum* which is representative for the grass quality used by grazing ruminants in Brazil (ABDALLA et al., 2012). The diet consisted of 70% of Tifton hay (*Cynodon*. *spp.*), 21% ground corn and 9% soybean meal.

The animals were randomly divided into three experimental diets completing a double Latin square design (3 treatments, 3 periods, 6 animals). In each experimental period, the animals were adapted to their treatments for 10 d followed by 7 d of collections. The sheep were housed in individual metabolic cages to assess the nutrients digestibility, nitrogen balance and rumen fermentation characterstics.

The animals were fed *ad libitum*, one of the dietary treatments: control (CNTRL - basal diet only), leucaena diet (LEUC -123 and 8.8 g/kg DM of total tannins and condensed tannins, respectively) which consisted of the basal diet replaced by 50% of Tifton hay with leucaena and the leucaena diet plus 20g/day/animal of polyethylene glycol (PEG MW 6000, Merck Schuchardt OHG, Hohenbrunn, Germany) (LPEG). Mineral premix and fresh water were offered *ad libitum* during all the experimental periods.

5.2.3 Feed intake, apparent total tract digestibility and N balance

The metabolic cages used for the measurements of nutrients digestibility and N balance were equipped for individual daily collection of feed refusals, faeces and urine. Loss of ammonia from urine was prevented by daily addition of 100 ml of 10% sulphuric acid to the collection flask (CHEN; GOMES, 1992).

Samples of the feed offered and refusals, faeces and urine were collected daily from each animal and stored at -20 ºC. At the end of the collection period, feed refusals, faeces and urine samples were pooled for each animal and representative samples were taken for analysis. Feeds offered, refusals and faeces samples were dried in a forced air-oven at 50ºC for 48 h and ground to pass a 1 mm-screen and stored at -20°C for analysis. Urine samples were frozen at – 20 ºC until analysed. The N balance was calculated as:

$$\text{N balance} = \text{N intake} - (\text{faecal N} + \text{urinary N})$$

5.2.4 Ruminal fluid sampling

During the collection period, rumen fluid (±250 ml) was sampled through the ruminal cannulae for two consecutive days, three hours after the morning feeding. Rumen fluid was filtered through three layers of cheesecloth and immediately analysed for pH. After filtration, 2 ml of rumen fluid was mixed with 4 ml of methylgreen–formalin saline solution (MFS) for microscopic protozoa count according to the procedure described by Dehority et al. (1983).

The method of Palmquist; Conrad (1971) was used for the determination of the rumen SCFA concentration with some modifications. Rumen fluid was centrifuged at 15.000 g for 10 min at 4 °C to remove large feed particles. 1.6 ml of the supernatant was centrifuged (15.000 g for 15 mints at 4 °C) after adding 0.4 ml solution of meta phosphoric acid (3:1) 25% and formic acid 98-100% plus 0.2 ml of 2-ethyl-butyric acid 100 mM (internal standard, MW = 116.16; Sigma Chemie Gmbh, Steinheim, Germany). After centrifugation, approximately 1.2 ml was transferred to the chromatographic vial. One µl was injected in to the gas chromatography [(CG HP 7890A; Injetor HP 7683B, Agilent Technologies, Palo Alto, CA, EUA Column capillary, HP-FFAP (19091F-112; 25 m; 0,320 mm; 0,50 µm; J&W Agilent Technologies Inc.; Palo Alto, CA, EUA)].

The calibration standard curve was prepared by known concentrations of external chromatographic standards (Chem Service, West Chester, PA, EUA) as following, acetic acid, (99.5%, CAS 64-19-97), propionic (99%, CAS 04/09/79), isobutyric (99%, CAS 79-31-2), butyric (98.7%, CAS 107-92-6), isovaleric (99%, CAS 503-74-2) and valeric (99%, CAS 109-52-4).

Total NH_3-N contents were measured in the rumen fluid supernatant according to Preston (1995) using micro-Kjeldahl by steam distillation with sodium tetraborate solution (5%), collected in boric acid solution (20%) and determined by titration with 0.05 N H_2SO_4.

5.2.5 Laboratory analyses

The feeds offered, refusals and faeces were chemically analyzed on dry matter (DM) basis according to AOAC (2006) as for organic matter (OM); crude protein (CP as 6.25×N) and ether extract (EE). The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were measured sequentially using the same sample in filter bags and expressed exclusive of residual ash according to Van Soest (1991) and adapted to Mertens (2002). The NDF were assayed with a heat stable amylase and ADL was determined by solubilization of cellulose with sulphuric acid (72%). Nitrogen in urine was determined by the Kjeldahl N method (AOAC, 2006).

5.2.5.1 Total phenols

The experimental diets (200 mg) were extracted with acetone 70% in an ultrasonic bath for 20 min. The contents were centrifuged at 4 °C for 10 min at 3,000 × *g* and the supernatant was kept on ice until analysis. Total phenols were determined with the Folin–Ciocalteau reagent and detected using spectrophotometer at 725 nm (MAKKAR et al., 1993; MAKKAR, 2003). A calibration curve was prepared using increment concentrations as 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10 ml of stock solution of tannic acid (0.1 mg/ml) (Merck GmbH, Darmstadt, Germany). Total phenols were calculated as tannic acid equivalents and expressed as eq-g/kg DM.

5.2.5.2 Total tannins

Total tannins (TT) were estimated by precipitating tannins with polyvinyl polypyrrolidone (PVPP, FW111.1, Sigma-Aldrich Inc., St. Louis, USA) according to Makkar et al. (1995). One hundred mg of PVPP was taken in test tube and then 1000 µl of distilled water and 1000 µl of tannins extract (previously discribed) were added.

The mixture was kept after vortex in refrigerator for 15 min. at 4 °C then centrifuged (7000 g for 10 min.at 4 °C). 1000 µl of supernatant was taken for estimation of non-tannins phenol in another test tube and 400 µl with distilled water was added and then processed like that of total phenol estimation with the Folin–Ciocalteau reagent. Extractable tannins were determined as the difference in total phenolics (measured by Folin–Ciocalteau reagent) before and after treatment with PVPP and were expressed as tannic acid equivalents based on DM.

5.2.5.3. Condensed tannins determinations

Condensed tannins (CT) were measured by the HCl-butanol method according to Makkar (2003). Half ml of tannins extract was taken in test tube in triplicate and 3.0 ml of butanol and 0.1 ml of ferric ammonium sulphate were heated in a boiling water bath for 60 min after covered the tubes with glass marble. Similarly blank was prepared for each sample but without heating the reagent. After cooling the tubes, absorbance was read at 550 nm and CT was were expressed as leucocyanidin equivalent.

5.2.5.4 Mimosine and 2,3- dihydroxypyridine (2, 3 DHP)

Leucaena mimosine and 2, 3 DHP were extracted using 0.1N HCl for HPLC analysis according to Wu et al. (2012). The HPLC equipment used was SHIMADZU (LC-6AD) system with binary pump, Agilent ZORBAX ODS column in conjunction with a Agilent ODS precolumn (5µm, 12.5 x 4.6 n.m I.D) at 35°C was used. Mimosine standard (Purity ≥ 98%) and 2, 3 DHP standard (Purity≥98%) were obtained from Sigma. The leucaena diets samples were eluted with a mobile phase of 0.2 (W/V) orthophsphoric acid and methanol. The optimal separation of the components was achieved at 265 nm, the limit of detection was 6.88 µg/ml and the limit of quantification was 20.85 µg/ml.

5.2.6 *In vivo* CH_4 assay

At the end of the digestibility trial, the animals were individually kept for two consecutive days in the respiratory chambers, which were tested for their suitability by infusion of known concentrations of CH_4 into the chambers and resulted in 107±9% recovery (data not reported).

Each chamber [157 length ×71 width ×167 height cm (volume 1.9 m3)] as described by Abdalla et al. (2012) was covered on sides, except the bottom, with 0.3-mm thick polyethylene sheets. The chamber has one inlet 5-cm orifice in the front and one outlet 5-cm orifice in the rear. An exhaust pump was connected to the rear orifice in order to remove the inner air at a flow rate of 168 L/min (measured using an anemometer—CE Instrutherm AD-250, Sao Paulo, Brazil). The outlet air was sampled into a 5L balloon (coated with aluminum film) at 100 ml/min using a peristaltic pump. A household fan was placed inside the chamber, for circulating air, to keep temperature and carbon dioxide at levels comfortable to the animal.

Temperature, humidity and air flow through the chamber were measured for 22 h, at regular intervals of 2 h in two consecutive days. Methane in the outgoing sampled air was evaluated by using a gas chromatograph (Shimadzu GC-2014, SINC Brazil, Sao Paulo, Brazil).

5.2.7 Statistical analysis

The data were statistically analyzed using a double Latin square (3 treatments, 3 periods, 6 animals) by SAS software (SAS, 2002) using the following model:

$yijk = \mu + Ti + Aj + Pk + eijk$

Where: $yijk$ = dependent variable;

μ = general mean;

Ti = treatment effect (i = 1 to 3);

Aj = animal effect (j = 1 to 6);

Pk = period effect (k = 1 to 3);

$eijk$ = error

Differences between treatments were declared significant at $P \leq 0.05$ using the Tukey correction for multiple comparisons.

5.3 Results

5.3.1 Feed composition

Nutrient ingredients and chemical composition of the experimental diets are given in Table 5.1. Leucaena diets had a relative high content of CP, ADL, TP, TT, CT and mimosine compared with the control basal diet, while there was a similarity in the NDF and ADF content for all the experimental diets. Generally, PEG supplementation for leucaena diet did not show changes on chemical composition.

Table 5.1 - Ingredients and chemical composition (g/kg DM) and tannin content of the experimental diets

Item	Control	Leucaena diets	
		Without PEG	With PEG
Ingredients (g/kg DM)			
Tifton hay	700	350	350
Leucaena	-	350	350
Ground corn	210	210	210
Soybean meal	90	90	90
Chemical Composition (g/kg DM)			
Organic matter	950	943	946
Crude protein	139	190	191
Neutral detergent Fiber	688	647	655
Acid detergent fiber	367	353	357
Lignin	87	128	127
Ether extract	35.2	35.9	36.2
TP	2.1	182.7	182.5
TT	1.05	122.5	122.2
CT	-	8.75	8.80
Mimosine	-	1.80	1.80
2,3-Dihydroxypyridine	-	Not detected	Not detected

PEG= polyethylene glycol, TP=Total phenols (eq-g tannic acid/kg DM), TT=Total tannins (eq-g tannic acid/kg DM), CT=Condensed tannins (eq-g leucocyanidin/kg DM).

5.3.2 Feed intake and diet digestibility

Table 5.2 presents the dry matter (DM) intake and the total tract digestibility of the nutrients. In the current study, DM intake was improved (P=0.009) by leucaena diets (LEUC and LPEG) compared with the control diets without a significant effects between leucaena diets. Based on kilogram of DMI the animals fed LEUC received daily 12.95 and 181 g/kg DMI of CT and TT, respectively, while the animals that fed LPEG received daily 13.55 and 188 g/kg DMI of CT and TT respectively.

Table 5.2 - Effect of replacing leucaena with or without polyethylene glycol (PEG) on feed intake and total tract diet digestibility

Response	Leucaena diet			*P*-value	S.E.M
	Control	Without PEG	With PEG		
Intake (g/day/sheep)					
Dry matter	1249[b]	1480[a]	1540[a]	0.009	153.001
Digestibility (%)					
Dry matter	57.06	55.58	57.02	0.772	4.420
Organic matter	59.03	58.11	57.85	0.766	3.191
Crude protein	64.98	62.03	66.22	0.086	3.300
Neutral detergent fiber	54.38	51.49	53.02	0.635	5.687
Acid detergent fiber	47.18[a]	38.45[b]	40.93[ab]	0.009	4.460
Ether extract	65.53	58.72	58.14	0.197	8.079

[a,b,c] Means within a row without a common superscript letter differ significantly ($P<0.05$)
S.E.M standard error mean.

Apparent total-tract digestibilities of DM, OM, CP, NDF, and EE were not affected (P>0.05) for all the experimental diets. However, LPEG diet tended to had a beneficial effect on CP digestion (P=0.086) by 6.75% compared with LEUC diet. For the ADF digestion coefficient, control diet showed the highest (P= 0.009) values compared with the leucaena diets.

5.3.3 Nitrogen balance

Nitrogen intake, excretion and retention are presented in Table 5.3. Leucaena diets (LEUC and LEPG) increased significantly (P= 0.0051) N intake compared with the control diet. Leucaena diets with or without PEG increased (P=0.0002) the faecal N execration compared with the control diet. In contrast, urine N excretion remained unchanged (P>0.05) among the experimental diets. While both of leucaena diets improved (P=0.012) the N retention compared with the control.

Table 5.3 - Effect of feeding leucaena diet with or without polyethylene glycol on N intake, N excretion and N retention

Response	Control	Leucaena diet		*P* -value	S.E.M
		Without PEG	With PEG		
N intake (g/sheep/day)	31.05[b]	44.73[a]	44.25[a]	0.0051	7.161
N (g/sheep/day)					
Faeces	10.40[b]	17.683[a]	15.900[a]	0.0002	2.248
Urine	12.41	10.78	10.50	0.435	2.941
Nitrogen retained (g/sheep/day)	10.56[b]	16.83[a]	17.83[a]	0.012	3.991

[a,b,c] Means within a row without a common superscript letter differ significantly ($P<0.05$)
[S.E.M] standard error mean.

5.3.4 Methane emission, fermentation parameters and protozoa count

Table 5.4 shows the effect of the PEG addition for leucaena diet on CH_4 emission, rumen fermentation parameters and protozoa count. Leucaena diets (LEUC and LPEG) decreased CH_4 production by 10.8 and 14.1% based on the digested OM respectively, compared with the control. Addition of PEG increased ($P<0.0001$) the rumen NH_3-N concentration by 19% compared with the control diet, while no significant differences were detected between the LEUC and control diet. Leucaena diets (LEUC and LPEG) did not affect ($P>0.05$) either rumen pH or protozoa count.

Compared with the control, LEUC diet decreased ($P= 0.012$) the acetate, butyrate ($P=0.0034$) and C2: C3 ratio ($P<0.0001$), whereas no significant differences were detected for molar proportions of C5, IC4, IC5 or total SCFA for all the diets. LEUC and LPEG diets increased ($P=0.0327$) the molar proportions of C3 by 10.6% and 17.8, respectively compared with the control diet.

Table 5.4 - Methane (CH_4) emission, fermentation parameters and protozoa count in the rumen fluid of sheep fed on leucaena diet with or without polyethylene glycol (PEG)

Response	Control	Leucaena diet		*P* -value	S.E.M
		Without PEG	With PEG		
CH_4 (L/day)	43.30	38.39	39.82	0.328	5.790
CH_4 (L/kg DOM)	55.18[a]	47.42[b]	49.24[b]	<0.0001	2.501
NH_3-N (mg/100ml)	20.44[b]	19.60[b]	24.32[a]	<0.0001	1.352
pH	6.39	6.50	6.34	0.220	0.1622
SCFA(mmol/L)					
C2	48.49[a]	40.43[b]	44.63[ab]	0.0122	4.430
C3	9.69[b]	10.72[ab]	11.42[a]	0.0327	1.100
C4	6.71[ab]	5.61[b]	7.44[a]	0.0034	0.881
C5	6.41	6.24	7.03	0.313	0.919
IC4	6.18	5.69	6.93	0.090	0.952
IC5	5.99	6.30	7.15	0.118	0.975
Total	83.49	75.01	84.63	0.0515	7.155
C2:C3	5.04[a]	3.81[b]	3.96[b]	<0.0001	0.345
Protozoa (10^5/ml)	6.25	6.70	6.88	0.544	1.100

[a,b,c] Means within a row without a common superscript letter differ significantly ($P<0.05$)
S.E.M standard error mean.
SCFA= short chain fatty acids, C2= acetate, C3=propionate, C4= butyrate, C5=valeriat, IC_4= iso butyrate, IC5= iso valeriat C2:C3, acetate, propionate ratio.

5.4 Discussion

The higher CP intake of leucaena diets (LEUC and LPEG), associated to its higher CP content compared with the control diet which confirmed its potential as protein supplement to improve the feed quality of ruminants that could help to improve productivity of ruminant in tropical regions (YUANGKLANG et al., 2011).

Lima et al. (2011) reported that tanniniferous leguminous presents not only high protein content but also high lignin. In our study, the higher lignin content of leucaena diet compared with the control could be due to leucaena was collected during the dry season. Lima et al. (2011) observed that the fresh forage available during the dry season showed higher ADF and lignin content than fresh forage available during the rainy season. In addition, leucaena stems participated as 13% from the whole leucaena plant which could have increased the lignin content.

Longo et al. (2008) reported that CT concentrations of the experimental leucaena diets were 5.5, 3.7 and 1.9 (g/kg DM) when leucaena sun dried was included at 60, 40 and 20% of Tifton basal diet. In the current study, leucaena included by 35% provide 8.77 (g/kg DM) CT

when using the same method of CT determination as the pervious study. This variability confirming that the CT content of leucaena diets depend on factors such as origin, storage, and drying systems (YÁÑEZ RUIZ et al., 2004).

Rochfort et al. (2008) demonstrated that PEG addition increased DM intake (DMI) when sheep were fed CT containing diets. PEG prevents binding of CT to protein thus the effect may be due to polyphenolic plant metabolites. However, the current results suggested that PEG addition to leucaena did not affect the DMI and such improvement in the DMI for the leucaena diets (LEUC and LPEG) compared with the control without adverse effect on the DM digestibility may be related to higher palatability of leucaena diet which may be supported by the higher CP content. Leucaena CT level in the current study was not high enough and could have a beneficial nutritional effect.

Mueller-Harvey (2006) reported that condensed tannins may be beneficial in the diet but at certain levels begin to affect feed intake. This level varies considerably, depending on the chemical nature of tannins and the animal species studied.

Our results are agreed with previously reported studies by Haque et al. (2008) who observed a linear decrease (P=0.04) of DM maize intake with increasing level of leaves and twigs of *Leucaena leucocephala* by goats indicated that the palatability of leucaena might have been better than maize. Hulman and Preston (1981) have showed that feed intake increased linearly with increased leucaena level resulting with a high correlation ($r2$ = 0.98) between the level of leucaena and total feed intake. Similarly, Longo et al. (2008) demonstrated that the high level of leucaena supplementation (40 and 60%) increased feed acceptability by Santa Inês sheep and CP intake due to the higher digestible nutrients compared to the Tifton based diet.

Apparent digestibility of DM, OM, NDF and EE were not significantly affected by PEG addition to leucaena diet, however the CP digestion tended to increase (P=0.086) by 6.75% compared with LEUC and increased significantly the NH_3-H concentrations of the rumen fluid. It would seem that protein binding of leucaena CT protected leucaena CP from the rumen degradation and such these decreasing in ruminal protein degradation may be supported by lower concentrations of ammonia in ruminal fluid of LEUC that may occur due to the formation of tannin–protein complexe in the rumen pH and inhibition of the activities of proteolytic bacterial populations (PATRA; SAXENA, 2011).

It seem that, the high lignin content of leucaena diets generally affect the ADF digestibility however, there was no significant differences between the LEPG and the control in ADF digestibility while LEUC decreased (P=0.009) the ADF digestibility compared with

the control. These results suggested that the fiber digestion increased by PEG addition, possibly because sufficient rumen degradable protein (RDP) was available in the rumen due to tannins inactivated by PEG. It is possible that soluble carbohydrates in the rumen may decrease as it was binding with PEG, this condition may be suitable to optimize cellulolytic microbial activity (JETANA et al., 2011).

Also, another reason which could explain the decreasing in ADF digestibility for leucaena diet was that the separation between the effects of lignin and tannin can be difficult because tannin and tannins-protein complex may appear as lignin or natural detergent in soluble nitrogen and therefore may apparently increase the content of ADF in plant and faecal samples (MAKKAR et al., 1995).

The effects of PEG supplementation and CT on nutrients digestibility in ruminants have been inconsistent among studies. Ben Salem et al. (2005) found that supplementing goats with 20 g/ day PEG to appropriate level of barley, i.e. 300 g/day and ad libitum kermes oak (*Quercus coccifera* L.) increased the CP digestibility. Similarly, Jetana et al. (2011) demonstrated that the digestibility of Thai swamp buffalo of CP and fiber were improved significantly by addition of 50g/day PEG to diet of rice straw supplemented with 500 g leucaena compared with the basal diet of rice straw.

In contrast, Yildiz et al. (2005) observed that inclusion of *Quercus hartwissiana* (oak) leaves at two levels of intake (185 and 370 g/day), together with two levels of dietary PEG (50 or 100 g/kg of dry oak leaves), in the diet of Tuj lambs reduced significantly CP digestibility and N retention thus PEG supplementation would be unnecessary with up to 370 g/day of oak leaves in the diets of pre-pubertal lambs. These varied results probably reflect different dietary levels of CT, as well as variation among plants in CT biological activity (MIN et al., 2003).

Logically higher CP content of leucaena diets increase the N intake compared with the control (Table 5.3). The N retention improvement which associated with the increasing in the faecal N execration instead of urinary N by both two types of leucaena diets is beneficial environmentally because urinary nitrogen is largely in the form of urea, which is more rapidly hydrolysed to ammonia and nitrified to nitrate (ECKARD et al., 2010). The nitrate could leach into ground water causing water pollution, and is also converted to nitrous oxide (a greenhouse gas) accounting for about 65% of global anthropogenic nitrous oxide emissions (ECKARD et al., 2010).

Secondly, a CT–protein complex in faeces dissociates slowly in the soil because mineralisation of the complex is inhibited, and the faeces decompose more slowly compared

with faeces without CT (ECKARD et al., 2010). Therefore, decreased nitrogen excretion in the urine could reduce ammonia and nitrous oxide emissions into the atmosphere (PATRA; SEXENA, 2011).

Similarly to our findings, Jetana et al. (2011); Ben Salem et al. (2000); Carulla et al. (2005) reported similar results of decreased N excretion in urine with subsequent decreases in rumen ammonia concentrations when sheep and goats were fed legumes that contained tannins.

Leucaena forages and leucaena tannin extracts have been demonstrated to decrease CH_4 emission both *in vivo* and *in vitro* studies. Tan et al. (2011) show a reduction (33%) in *in vitro* CH_4 production at even the lowest inclusion of 10 mg Leucaena CT/500 mg DM, but the reductions were more (up to 63%) at higher CT levels. However, unexpectedly in the current study, both of leucaena diets (LEUC and LPEG) decreased the CH_4 emission compared with the control diet.

Sallam et al. (2010) found a similar significant (P<0.05) decrease in CH_4 production *in vitro* for two tanniferous legumes leaves, leucaena (*Leucaena leucocephala*) and acacia (*Acacia saligna)* containing 32.5 and 61.4 CT eq-g leucocyanidin/ kg DM respectively, by 88 and 89 % respectively compared with alfalfa as a non tannin control diet while PEG supplementation resulted in increase of gas production (GP) by 28.1 and 87.4 % for leucaena and acacia, respectively compared with alfalfa. In Sallam et al. (2010) study, if the CH_4 reduction was related to the leucaena CT so both of leucaena and acacia would have the same increase of the GP by PEG addition that because they are similar in their CH_4 reduction but the increase in GP was 67.8% higher for acacia compared with leucaena.

These results of Sallam et al. (2010) and our findings suggest that factors other than leucaena CT could explain the reduction of CH_4 emission in the presence of PEG of leucaena diet. However leucaena tannins deactivation by PEG administration can give a chance for its free toxic mimosine amino acid to be studied (AKKARI et al., 2008; YAMI et al., 2000) but unfortunately, the current study could not confirm the antimethanogenic properties of leucaena mimosine because we did not examine the effect of mimosine supplementation for both diets. Moreover, to our knowledge, no study has examined the leucaena mimosine effect on CH_4 emission but such changes may be attributable to characteristics of the two forage types [(*i.e.*, legume (leucaena) compared with grass (Tifton)].

Factors responsible for differences among findings are unclear but may include variation in chemical composition in legumes and grasses and associated microbial activity and rate of digesta outflow from the rumen (CARULLA et al., 2005). Pinares-Patiño et al.

(2003) noted that CH_4 emission by sheep grazing perennial ryegrass-based pasture, lucerne, and a CT-containing legume *L. corniculatus* was 0.08, 0.05, and 0.03 of GE intake, respectively, and concluded that CT in such forages are not solely responsible for differences in CH_4 emission compared with grass diets.

Ungerfeld et al. (2003) and Tan et al. (2011) demonstrated that the rumen fermentation manipulation of CH_4 production is a means of diverting H_2 away from CH_4 formation. Therefore an alternative electron sink metabolic pathway to dispose reducing power has to occur. Newbold et al. (2005) reported that the succinate propionate pathway that leads to propionate production could be the best alternative metabolic pathway. Hence, an increase in propionate production would be expected if propionate formation has become an alternative pathway for H_2 disposal in the rumen. In these sense, decreasing acetate and butyrate and increasing propionate production which is associated with a decreasing in CH_4 emission with the constant concentration of total SCFA of LEUC treatment are sufficient to indicate such this pathway occurred in the presence of leucaena tannins.

Although, LPEG had the highest propionate production which associated with the decreasing in CH_4 emission but also improved the acetate and butyrate production, these results shows that the PEG addition allowed more fermentable nutrients to be fermented in the rumen which supported partially with the improvement in the ADF digestibility by 6.45% compared with LEUC (CARULLA et al., 2005).

An increase in acetate production associated with the cellulose (major part of ADF) digestibility would be due to increased acetogenesis, a CO_2-reducing process to produce acetate, which is also a way to dispose of metabolic H_2 during microbial metabolism. However, acetogenesis is thermodynamically less favorable than methanogenesis (MCALLISTER; NEWBOLD, 2008) and acetogens have a poorer affinity for H_2 than methanogens and it seems that the major butyrate producer, *Butyrivibrio fibrisolvens* was not inhibited by PEG addition. Moss et al. (2000) reported that acetate and butyrate promote methane production while propionate formation can be considered as a competitive pathway

for hydrogen use in the rumen. Thus it could be explain why LEUC decreased CH_4 by 14.06 % compared with the control while LPEG decreased CH_4 by 10.76 % compared with the control.

Tannins have been shown to lower protozoal numbers, which may also decrease protozoal-associated methanogenesis (PATRA; SAXENA, 2011; SOLTAN et al., 2010). Hess et al. (2003) also reported that CT-containing legumes showed methanogenic toxicity. Recently, Tan et al. (2011) demonstrated that total protozoa using real-time PCR assay also revealed a reduction in the protozoal population when leucaena CT inclusions above 10 mg/500 mg were added *in vitro*. However such effect did not occurred in the present study. Similarly to our findings, Benchaar et al. (2008) did not observe any effect on protozoal numbers in dairy cattle fed quebracho tannins (CT concentrations of 700 g kg−1, 150 g day−1) probably due to addition of a low dosage in the diet. The reason for this discrepancy may be due to the different in CT activity, and/or its concentration.

5.5 Conclusions

The partial replacement of Tifton hay by leucaena with or without PEG improved the DM, CP intake as well as deceased (P<0.0001) the CH_4 emission (relative to OM digested) by 14.1 and 10.8 % for LEUC and LPEG compared with the control Tifton basal diet without adverse effect on the total tract nutrients digestibility.

Both the LEUC and LPEG diets decreased the C2/C3 ratio. However LPEG diet improved the acetate and butyrate compared with the LEUC diet such these results pointed out the availability of more fermentable nutrients by PEG addition.

PEG addition should not be offered to animals fed on low CT diets since its addition had not carry-over effect for the methane emission.

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6 GENERAL CONCLUSIONS

- The chemical composition of the tanniniferous plants (*i.e.* leaves of prosopis (*Prosopis juliflora),* acacia *(Acacia saligna),* atriplex *(Atriplex halimus),* and leucaena *(Leucaena leucocephala*) confirming their potential as additives for application for ruminants to overcome Feedstuff's shortage in the tropical regions.
- Highly tanniniferous plants like acacia and leucaena (63 and 46 eq-g leucocynidin /kg DM, respectively) protect the dietary protein from rumen microbial degradation with diferences in the intestinal protein digestibility.
- The potential methanogenic properties of tanniniferous browses may be not only related to the tannins content but also to the tannins activity and nutrients composition.
- Both prosopis and leucaena presented similarity in their rumen fermentation responses patterns by decreasing acetate: propionate ratio with increasing propionate although prosopis had negligible CT content compared with leucaena.
- Essential oils active components could be used as natural alternatives modifiers of rumen microbial fermentation to decrease CH_4 production since moderated supplementation levels (at 10 and 20 µl/75 ml of culture fluid) of carvacrol and eugenol showed a similarity with monensin in their effect on CH_4 reduction and rumen organic matter degradability with different mode of action.
- The mode of action of decreasing methane production by carvacrol and eugenol is different from monensin since monensin increase the propionate concentration and decrease the acetate: propionate ratio but both carvacrol and eugenol increase the butyrate concentrations without effect on the total SCFA.
- In contrast with highly tannin tanniniferous plants, the phenolic essential oils active components did not affect on the rumen NH_3-N, suggesting that deaminative activity of ruminal bacteria was not affected by these components.
- The effect of the anti protozoal activity that associated with the lower methanogenesis was appeared with the essential oils active components supplementations while was not found for the tanniniferous plants either in *in vitro* or *in vivo* studies.
- The increasing in the crud protein content of the Tifton hay based diet replaced partly by leucaena confirmed its potential as protein supplement to improve the feed quality of sheep diets.

- Leucaena diet (123 and 8.8 g/kg DM of TT and CT respectively) either with or without PEG decreased CH_4 emission *in vivo* without adverse effect on the nutrients intake or the apparent total tract digestibility as well as decreased the acetate: propionate ratio compared with the Tifton hay based diet.
- The associated improve of the N retention and increase the faecal N execration instead of the urine of animals fed on leucaena diet reflecting the beneficial environmental by reducing ammonia and nitrous oxide emissions into the atmosphere.
- Concerning to the CH_4 reduction in the leucaena diet *in vivo* either with or without PEG addition, it could be concluded that PEG should not be offered to animals fed on low CT diets since PEG addition had not carry-over effect.
- Methane mitigation strategy using the tanniniferous plants and essential oils studied showed potential to suppress methanogenesis both *in vitro* and *in vivo*, however, the overall objective of reducing greenhouse gases may not be entertained unless evaluated the entire life cycle of these strategies.