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Mitigating the greenhouse gas balance of ruminant production by identifying plants with high tannin concentration and quantifying the methane emission *in vivo*

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நீங்கள் ஒரு சூரியனைப்போல் பிரகாசிக்க வேண்டும் என்றால்,
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If you want to shine like a sun, first burns like a sun.

-Dr. A. P. J. ABDUL KALAM
11th President of India.

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ABSTRACT

DINESHKUMAR, D. **Mitigating the greenhouse gas balance of ruminant production by identifying plants with high tannin concentration and quantifying the methane emission *in vivo.*** 2016. 102 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2016.

In Brazil, with the continued expansion of agriculture for supplying demands from international markets, progressive increases in emissions of greenhouse gases are expected. The purpose of the project was hypothesized with three major approaches, 1) Strategies to mitigate methane emission in small ruminant production systems; 2) Identify tropical plants and individual bioactive compound against methanogenic propertie and 3) *In vivo* evaluation of the nutrients metabolism in Santa Ines sheep fed tropical plants. For this, we have performed three experiments. The first study (**Expt. 1)** was designed to determine the *in vitro* effects of three tropical tannin rich plants such as *Leucaena leucocephala* (LL), *Mimosa caesalpiniifolia* (MC), *Schinus molle* (SM) and one non-tannin rich plant *Medicago sativa* (MS) for their anti-methanogenic properties when used with and without polyethylene glycol (PEG). All plants had significantly ($P<0.05$) influenced the degraded organic matter (TDOM) and degraded neutral detergent fiber (DNDF), especially LL, which had most influence on these parameters compared to other tannin containing plants. LL had positive response on antimethanogenic effects; its nutrient degradability was higher than that of other tannin containing plants. The second study (**Expt. 2**) was set to evaluate the effect of different organic extracts from the whole plant methanolic extract (MHE) of LL on *in vitro* gas production and to characterize the chemical constituents by using gas chromatography coupled with mass spectroscopy (GC-MS). Major abundant compounds present at the relative percentages of MHE were found to be stigmasterol trimethyl ester (TMS), neophytadiene, palmitic acid TMS, α-Linolenic acid TMS and 2, 3, 5, 6-tetra methyl anisylbenzene. The effects of additions of different extracts in terms of nutrient degradability (TDOM and DNDF) were increased by all extracts. This study explained that the methanolic hexane extract and methanolic chloroform extract was effective against methanogenic activity. The objective of the third study (**Expt. 3)** was to study the effect of LL plant leaves on rumen fermentation, apparent nutrient digestibility, nitrogen balance and methane production in Santa Ines sheep. The animals were divided in three groups in which they were fed with (i) 88% Tifton 85-hay (*Cynodon spp.*) and 12% soyabean meal (*Control group*, n=4); (ii) 28% Tifton 85-hay (*Cynodon spp.*) and 72% LL plus 20 ml solution containing 10g/day/animal of PEG (*With PEG group* - WPEG, n=6); (iii) 28% Tifton 85-hay (*Cynodon spp.*) and 72% LL plus 20 ml of distilled water (*without PEG group*- WOPEG, n=6). Nutrient intake (dry matter, organic matter, acid detergent fiber, lignin and crude protein) were higher in WPEG and WOPEG compared to the control group, except neutral detergent fiber intake. Apparent digestibilities and nitrogen metabolism had non-significant effects between the treatments. However, CH_4 emissions were significantly lower in WPEG and WOPEG than the control. Furthermore, expressions of microbial populations of methanogens in WPEG had lower tendency than that of WOPEG and control. The most salient findings of this study were that, 72% LL plant leaves using in small ruminants diets increased animal productivity, we can get more benefits in terms of replacing the source of protein in the diet (food safety) and reduced production of enteric CH_4 (animal production).

Keywords: *In vivo. Leucaena leucocephala.* Anti-methanogenic properties. Food security.

RESUMO

DINESHKUMAR, D. **Mitigando o equilíbrio de gases do efeito estufa na produção de ruminantes pela identificação de plantas com concentração elevada de tanino e quantificação das emissões de metano *in vivo*.** 2016. 102 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2016.

No Brasil, com expansão da agricultura para suprir as exigências dos mercados internacionais, são esperados aumentos progressivos nas emissões de gases do efeito estufa. O objetivo do projeto foi hipotetisado com três abordagens principais, 1) estratégias para mitigar emissões de metano em sistemas de produção de pequenos ruminantes; 2) identificar plantas tropicais com compostos bioativos com propriedades antimetanogénicas e 3) avaliação *in vivo* do metabolismo de nutrientes em ovelhas Santa Inês alimentadas com planta taninífera. Para isso, foram efetuados três experimentos. O primeiro estudo (Expt 1) foi concebido para estudar os efeitos *in vitro* de plantas tropicais ricas em taninos como *Leucaena leucocephala* (LL), *Mimosa caesalpiniifolia* (MC) e *Schinus molle* (SM) e uma planta não taninífera, *Medicago sativa* (MS) quanto às propriedades antimetanogénicas quando usadas com e sem polietileno glicol (PEG). Todas as plantas significativamente ($P < 0.05$) influenciaram na degradabilidade da matéria orgânica (MOD) e da fibra em detergente neutro (FDND), especialmente LL, que teve maior influência sobre estes parâmetros, em comparação com as outras plantas que contém taninos LL teve resposta positiva sobre os efeitos de antimetanogênicos e a degradabilidade dos nutrientes foi maior do que a das outras plantas que contém tanino. O segundo estudo (Expt 2) foi definido para avaliar o efeito de diferentes extratos orgânicos a partir do extrato metanólico da planta (EMP) de LL na produção de gases *in vitro* e caracterizar os constituintes químicos usando cromatografia gasosa acoplada com espectroscopia de massa (GC-MS). Os compostos mais abundantemente encontrados, em termos de percentagens relativas do EMP, foram o éster de trimetil estigmasterol, neofitadina, ácido palmítico, ácido α-linolênico e 2, 3, 5, 6-tetra methyl anizilbenzeno. Os efeitos de adições dos diferentes extratos orgânicos, em termos de degradabilidade de nutriente (MOD e FDND) foram aumentados para todos os extratos. Este estudo explicou que o extrato de hexano a partir do EMP foi eficaz na atividade anti metanogênicas em modificar a degradação ruminal de nutrientes. O objetivo do terceiro estudo (Expt 3) foi estudar o efeito das folhas da planta LL na fermentação ruminal, digestibilidade aparente de nutrientes, balanço de nitrogênio e produção de metano em ovinos Santa Inês. Os animais foram divididos em três grupos em que eles foram alimentados com (i) 88% feno de Tifton-85 (*Cynodon spp.*) e 12% de farelo de soja (Grupo controle, n = 4); (ii) 28% feno de Tifton-85 (*Cynodon spp.*) e 72% LL mais 20 ml solução contendo 10g/dia/animal de PEG (grupo com PEG - CPEG, n = 6); (iii) 28% feno de Tifton-85 (*Cynodon spp.*) e 72% LL mais 20 ml de água destilada (grupo sem PEG- -SPEG, n = 6). A ingestão de nutrientes (matéria seca, matéria orgânica, fibra em detergente ácido, lignina e proteína bruta) foram maiores no grupos CPEG e SPEG em relação ao grupo controle, exceto a ingestão de fibra em detergente neutro. As digestibilidades aparentes e o metabolismo do nitrogênio não apresentaram efeitos significativos entre os tratamentos. No entanto, as emissões de CH_4 foram significativamente inferiores nos grupos CPEG e SPEG em comparação com o grupo controle. Além disso, as expressões de populações microbianas de metanogênicas no grupo CPEG apresentaram tendência menor do

que nos grupos SPEG e controle. As conclusões mais relevantes do presente estudo foram que, usando 72% LL folha de planta em dietas de pequenos ruminantes, poderemos ter mais benefícios em termos de substituição da fonte de proteína da dieta (segurança alimentar) e redução da produção de CH_4 entérico.

Palavras-chave: *In vivo. Leucaena leucocephala.* Propriedades anti metanogênicas. Segurança alimentar.

LIST OF ABBREVATIONS

DM	Dry matter
OM	Organic matter
NDF	Neutral Detergent Fiber
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
CP	Crude Protein
TP	Total Phenol
TT	Total Tannin
CT	Condensed Tannin
MS	*Medicago sativa*
LL	*Leucaena Leucocephala*
MC	*Mimosa caesalpiniifolia*
SM	*Schinus molle*
PEG	Polyethylene Glycol
GP	Gas Production
PRO	Protoza
NH3-N	Ammonia Nitrogen
C2	Acetate
C3	Propionate
$C4_a$	Iso-butyurate
$C4_b$	Butyurate
$C5_a$	Iso-Valerate
$C5_b$	Valerate
C2/C3	Acetate/propionate ratio.
TSCFA	Total short chain fatty acids
CMAE	Crude Methanolic Alfafa Extract
CMLE	Crude Methanolic Leucaena Extract
MHE	Methanolic Hexane Extract
MCE	Methanolic Chloroform Extract
MEE	Methanolic Ethyl acetate Extract
MBE	Methanolic Butanol Extract
MRE	Methanolic Residue Extract
SCFA	Short Chain Fatty Acids
TDOM	Trully Degraded Organic Matter
PF	Partition Factor
GC-MS	Gas Chromatography Mass spectrophotometer
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
AOAC	Association of Official Analytical Chemists

TMS	Trimethylsilylester
CENA	Center for Nuclear Energy in Agriculture
CH_4	Methane
CNL	Control
WPEG	With PEG
WOPEG	Without PEG
PVPP	Polyvinyl Pyrrolidine

SUMMARY

1. INTRODUCTION

Climate change is one of the major threats on our planet with increasing population and also economical demand (SKUCE et al., 2013). According to the International Panel of Climate Change (IPCC) reported that rate of climate change is faster than never before in the last 1000 years and there is a possibility that the rise of average global temperatures between 1.8^0C and 4.0^0C within the next 90 years (YATOO et al., 2012). Hence, the impacts of global climate change is predominately threatening factors for the well-being of current and future generations (MARINO et al., 2015).

Livestock sector plays a very important contribution towards to the greenhouses gas emissions (GHGE) worldwide (i.e carbon-di-oxide (CO_2), methane (CH_4) from enteric fermentation and nitrous oxide (N_2O) from manure management (GERBER et al., 2011). Steinfield et al. (2006) estimated that this sector emits around 18% of total global anthropogenic GHG emissions. With increasing global population growth in developing countries, which demands more quantity of food products with lower environmental impact associated with their production. Hence, researchers are being focus on these aspects in the livestock sector (STEINFELD et al., 2006; GODFRAY et al., 2010). In particular, small ruminant sector plays a crucial worldwide socio-economic importance in terms of animal production and environmental performance. Sheep and goats represents about 56% on the global ruminant population. However, small ruminant populations are located in three different zones (56%-arid; 27%-temperate and 21%-humid) (MARINO et al., 2015). Foresight et al. (2011) reported that the expected rise of sheep numbers is around to be 60% by 2050. During past two decades, goats and sheep produce more than 28 million tons of milk and 13 million tons of meat respectively (FAOSTAT, 2013). Therefore, there is a strong interest in small ruminant sector, which is a very good model to evaluate the ruminant production systems in terms of animal performance and also measuring environmental impacts.

In Brazil, with the continued expansion of agriculture supplying demands from international markets, progressive increases in emissions of methane, nitrous oxide and carbon dioxide are expected. Enteric fermentation by ruminants, manure management and rice cultivation plus fuel burn of agricultural residues were accountable for 73%, 3% and 2% of total methane emitted in Brazil respectively (CERRI et al., 2009). Land use changes (19%) and industrial processes (3%) also accounted for the total 340 Mt CO_2 eq of Brazilian emissions (ABDALLA et al., 2012). Although most of the actual Brazilian livestock sector be

represented by cattle, a significant portion composed of small ruminants such as sheep and goats is in the Northeast states in Brazilian with different production systems, mostly extensive, based on grazing in the Caatinga, an important ecosystem of the semiarid region whose occupancy is approximately 60% of the area in Northeastern region.

In developing countries, animals have been an important factor in integrated livestock-crop farming systems. Animals have diversified role on production of animal protein and useful in farm manure as well as improving people livelihoods (WANAPAT et al., 2010). With expected global population growth increases around 8.3 billion people in the year 2030, it is essential to produce sufficient amount of food from locally available resources especially in developing countries. Level of consumption of animal food is increase from 10 kg/yr (1960) to 26 kg/yr (2000) and there is expected to rise up to 37 kg/yr in 2030 (FAO, 2008; 2009; WANAPAT et al., 2013). Most importantly, ruminant animals will continue as predominant factor on animal agriculture due to conversion of human inedible materials such as tree fodder, roughage, crop residue and by-products into human food. Hence, it is necessary to use locally available human inedible resources to increase animal productivity. Furthermore, Wanapat et al. (2009) reported that utmost importance of local available feed resources for ruminants to increase the animal production. There is a growing realization that mitigation action may not be isolated and it should be packed with increase in animal productivity and thus deliver against food security. Therefore, nowadays researchers are being focus on mitigation strategies and potentials that simultaneously improving animal productivity in terms of food security and livelihoods of farmers.

There are many mitigation practices used to reduce enteric CH_4 emissions and improve livestock productivity. However, several countries are restricting the availability of some mitigation options. For example, European Union banned antibiotics use in animal feeds due to human food safety (EUROPEAN UNION, 2003). Use of tannin containing plants have been studied and show the most promise for mitigating enteric CH_4 emissions. Beauchemin et al. (2007) reported that, tannin has potential for reducing enteric CH_4 emission by up to 20 percent. Tannins are being reported as anti-nutritional; at lower concentration it improves animal productivity in terms of alterations of ruminal fermentation and microbial protein synthesis (BHATTA et al., 2012).

Brazil has considerable territorial extension of the semi-arid northeastern Brazil is composed of savanna type natural grassland, characterized by different communities of plants, shrubs, trees, and little herbaceous. However, using polyethylene glycol (PEG) as a tannin binding agents forming tannin-PEG complexes have been used to determine the magnitude of

the tannin effects on rumen fermentation for certain plant species by *in vitro techniques*. It is a simple and effective tool, irrespective of their chemical nature. Hence, researchers had an opportunity to exploit plants and plant secondary metabolites as natural alternatives to improve livestock productivity.

Ruminant production in tropical region is generally based on forage grasses contain high amount of fiber and lignin content, which are being digested and loss of excessive energy to produce CH_4 and it represents loss of 8-12% gross energy (ABDALLA et al., 2012). In particular, higher CH_4 is produced, when diet based on grasses compared to the legumes based diets (GOEL; MAKAR, 2012). In this context, *Leucaena leucocepahala* (LL) has several attributes such as highly nutritious leguminous forages and it can provide firewood, shade and control in soil erosion. LL leaves contains high level of protein (which can able to replace soyabean meal in ruminants diets) and it also contains tannins (it could reduce CH_4 production). However, several studies reported that LL can be to improve ruminal fermentation in terms of metabolic protein supply due to its high protein content (SALLAM et al., 2010; SOLTAN et al., 2012). Similiarly, LL has been shown anti-methanogenic properties *in vitro* and *in vivo* (SOLTAN et al., 2012; 2013). In addition, compounds present in LL can improve rumen function with increase in number of microbial growth especially cellulolytic and proteolytic bacteria (HOOVER; STOKES, 1991; TAN et al., 2011).

Furthurmore, there are another mitigation options such as feed additives to improve livestock productivity. Feed additives are included into animal diets to improve feed quality, growth, nutrient deficiency, adsorb toxins, breakdown of anti-nutritive factors and reduce methane production in the rumen (DURMIC et al., 2014). Many researches in the last two decades have been focused on the effects of ionophores and antibiotics on ruminal fermentation (RUSSELL, 1987). In other hand, supplement of probiotics into the rumen microorganisms, which increase propionate or butyrate and reduce the protozoa number resulted reduction in methane (IQBAL et al., 2008). But, usage of probiotics in large scale production to mitigate CH_4 emissions is very expensive. Therefore, use of plant extracts containing high level of plant secondary metabolites will improve animal performance and resolve human safety issues. Several studies emphasized that group of plant secondary metabolites (e.g. saponins, flavonoids, and tannins) seems to present the ability to manipulate rumen fermentation in a favorable way thus lessening the CH_4 formation (HRISTOV et al., 2013).

The hypothesis of this work was the quantification of methane emissions in sheep under different feeding systems, using tropical plants or plant extracts as promising element to explain the use of tropical plants in ruminant diets in terms of food security and reduction of greenhouse gases.

The overall objective of the work was hypothesized with three main approaches: 1) strategies to mitigate methane emissions in small ruminant production systems; 2) identification of tropical plants and bioactive compounds with anti-methanogenic properties; 3) *in vivo* evaluation and performance of Santa Inês sheep fed with selected tropical plants.

The specific objectives were to determine the effects of tanniniferous tropical plant *Leucaena leucocephala* (*LL*), *Mimosa caesalpiniifolia* (*MC*) and *Schinus molle* (*SM*), for its anti-methanogenic properties when used with and without polyethylene glycol (PEG), as well as to evaluate the effect of different organic extracts from the methanolic extract of *LL* in upon the *in vitro* gas production and degradability and in addition, to characterize the chemical constituents using gas chromatography and mass spectroscopy (GC/MS). *In vivo*, the objective was to evaluate the effect of *LL* on ruminal fermentation, nutrient digestibility, nitrogen balance and emission of CH4 in Santa Inês sheep.

1.1. INTRODUÇÃO

As mudanças climáticas constituem uma das principais ameaças do nosso planeta com o aumento da população e também com a demanda econômica (SKUCE et al., 2013). O Painel Internacional de Mudanças Climáticas (PIMC) informou que a taxa da mudança climática é mais rápida dos últimos 1000 anos e que possivelmente haja um aumento das temperaturas médias globais em torno 1,8 e 4,0 °C nos próximos 90 anos (YATOO et al., 2012). Assim, os impactos dessa mudança climática global são, predominantemente, fatores que ameaçam o bem-estar das gerações atuais e futuras (MARINO et al., 2015).

A pecuária contribui expressivamente para as emissões de gases de efeito estufa (GEE) (tais como, dióxido de carbono - CO_2, metano - CH_4, proveniente da fermentação entérica e óxido nitroso - N_2O, proveniente do manejo de dejetos) em todo o mundo (GERBER et al., 2011). Com o aumento do crescimento populacional nos países em desenvolvimento, exige-se maior quantidade de produtos alimentares com menor impacto ambiental associado com a sua produção. Nesse sentido, os investigadores estão focados sobre estes aspectos no setor da pecuária (STEINFELD et al., 2006; GODFRAY et al., 2010). Em particular, o setor de pequenos ruminantes tem uma importância socioeconômica mundial crucial em termos de produção animal. Ovinos e caprinos representam cerca de 50% da população mundial de ruminantes. No entanto, esse efetivo está localizado em três diferentes zonas: 56% árida, 27% temperada e 21% úmidas (MARINO et al., 2015). Foresight et al. (2011) relatam um possível aumento de cerca de 60 % do rebanho ovinos em 2050. Durante as duas décadas passadas, caprinos e ovinos produziram mais de 28 milhões de toneladas de leite e 13 milhões de toneladas de carne, respectivamente (FAOSTAT, 2013). Portanto, há um forte interesse no setor de pequenos ruminantes, que é um bom modelo para avaliar os sistemas de produção em termos de desempenho animal e também a avaliação dos impactos ambientais.

No Brasil, com a contínua expansão da agricultura para o suprimento das demandas dos mercados internacionais, são esperados aumentos significativos nas emissões de metano, óxido nitroso e dióxido de carbono. A fermentação entérica de ruminantes, o manejo de dejetos e cultivo de arroz, mais consumo de combustível de resíduos agrícolas foram responsáveis por 73, 3 e 2 % do total de metano emitido no Brasil, respectivamente (CERRI et al., 2009). Mudanças no uso da terra (19 %) e processos industriais (3 %) também foram responsáveis pelo total de 340 Mt CO_2 eq das emissões Brasileiras em 2010 (ABDALLA et al., 2012). Embora a maior parte do setor da pecuária brasileira ser representado pelo gado,

uma parcela significativa composta de pequenos ruminantes, como ovinos e caprinos está na região nordeste do Brasil com diferentes sistemas de produção, principalmente o extensivo, com base em pastejo na Caatinga, um importante ecossistema da região semiárida, cuja ocupação é de aproximadamente 60 % da área do nordeste.

Em países em desenvolvimento, os animais têm sido um importante fator nos sistemas de integração lavoura-pecuária. Nessas regiões os animais ruminantes têm importante função na produção de proteína animal, assim como na melhoria de vida da população (WANAPAT et al., 2010). Com o esperado aumento do crescimento populacional, em torno de 8,3 bilhões de pessoas em 2030, é essencial produzir quantidade suficiente de alimento em regiões com recursos disponíveis, especialmente em países em desenvolvimento. O nível de consumo de alimentos de origem animal aumentou de 10 em 1960 para 26 kg/ano em 2000 e é esperando subir para 37 kg/ano em 2030 (FAO, 2008; 2009; WANAPAT et al., 2013). O mais importante é que, os animais ruminantes continuarão como fator predominante na produção animal devido à sua capacidade de converter alimentos não utilizados na alimentação humana como forragem, volumoso, resíduos de culturas e subprodutos em produtos de alta qualidade para alimentação humana.

Assim, é necessário usar recursos forrageiros localmente disponíveis para aumentar a produtividade animal (WANAPAT et al., 2009). Há uma percepção crescente de que as medidas de mitigação não podem ser isoladas e deve estar em conexão com o aumento da produtividade animal e, assim, oferecer uma maior segurança alimentar. Portanto, hoje em dia os pesquisadores estão focando em estratégias de mitigação e potenciais que simultaneamente melhoram a produtividade animal em termos de segurança alimentar e meios de subsistência dos agricultores.

Muitas práticas de mitigação foram utilizadas para reduzir as emissões de CH_4 entéricos. No entanto, vários países estão restringindo algumas dessas práticas. Por exemplo, a União Européia proibiu uso de antibióticos na alimentação animal devido à segurança alimentar humana (EUROPEAN UNION, 2003). O uso de plantas taniníferas tem sido estudado, mostrando ser muito promissor para na mitigação das emissões de CH_4 entérico. Beauchemin et al. (2007) relataram que o tanino tem potencial para reduzir 20 % da emissão de CH_4 entérico. Contudo, os taninos contém propriedades anti-nutricionais, mas em baixas concentrações pode melhorar a produtividade animal em termos de alterações da fermentação ruminal e a síntese de proteína microbiana (BHATTA et al., 2012).

A região semiárida do nordeste do Brasil possui uma grande extensão territorial composta por pastagem natural do tipo de savana, caracterizada por diferentes comunidades de plantas, arbustos, árvores e herbáceas. Pesquisadores têm buscado explorar as plantas e metabólitos secundários contidos nessas plantas como alternativas naturais para melhorar a produtividade animal.

A produção de ruminante em região tropical é geralmente baseada em forragens que contem alta quantidade de fibra, que ao serem digeridos, promovem excessiva perda de energia para produção de CH_4, representando de 8-12% de perda da energia bruta consumida (ABDALLA et al., 2012). Em particular, maior quantidade de CH_4 é produzida quando a dieta é baseada em forragens à base de gramíneas, quando comparado com dieta baseada em leguminosas (GOEL; MAKAR, 2012).

Nesse contexto, *Leucaena leucepahala* (LL) possue vários atributos, como ser altamente nutritiva, podendo fornecer lenha, sombra e controle da erosão do solo. As folhas de LL contém alto nível de proteína, que pode substituir a soja na dieta de ruminantes e contém também taninos, que podem reduzir a produção de metano entérico. Vários estudos reportaram que LL pode melhorar a fermentação ruminal em termos de fornecimento de proteína metabólica, devido ao seu alto teor de proteína (SALLAM et al., 2010; SOLTAN et al., 2012). Semelhantemente, LL tem mostrado propriedades anti-metanogênicas *in vitro* e *in vivo* (SOLTAN et al., 2012; 2013). Em adição, compostos presentes na LL podem melhorar a função ruminal com aumento no número de microrganismos, especialmente bactérias celulolíticas e proteolíticas (HOOVER; STOKES, 1991; TAN et al., 2011).

O uso de plantas ou extrato de plantas contendo alto nível de metabólitos secundários pode melhorar o desempenho animal e auxiliar nas questões de segurança alimentar. Vários estudos enfatizaram que metabólitos secundários de plantas (como as saponinas, flavonoides e taninos) têm a capacidade de manipular a fermentação ruminal favorecendo a redução da formação de CH_4 (HRISTOV et al., 2013).

Como hipótese para este trabalho destacamos a quantificação das emissões de metano em ovinos sob diferentes sistemas de alimentação, usando plantas tropicais ou extratos vegetais na dieta experimental como elemento promissor para explicar o uso de plantas tropicais em dietas de ruminantes em termos de segurança alimentar e redução de gases de efeito estufa.

O objetivo geral do trabalho foi hipotetisado com três abordagens principais: 1) estratégias para mitigar emissões de metano em sistemas de produção de pequenos ruminantes; 2) identificação de plantas tropicais e compostos bioativos com propriedades anti-metanogénicas; 3) avaliação *in vivo* de desempenho de ovinos Santa Inês alimentados com plantas tropicais selecionadas.

Os objetivos específicos foram determinar os efeitos das plantas taniníferas tropicais *Leucaena leucocephala* (LL), *Mimosa caesalpiniifolia* (MC) e *Schinus molle* (SM), por suas propriedades anti-metanogênica e quando usadas com e sem polietileno glicol (PEG), bem como avaliar o efeito de diferentes extratos orgânicos a partir do extrato metanólico da planta de LL na produção de gases e degradabilidade *in vitro*; além de caracterizar os constituintes químicos usando cromatografia gasosa e espectroscopia de massa (CG-EM). *In vivo*, objetivou-se avaliar o efeito da LL na fermentação ruminal, digestibilidade dos nutrientes, balanço de nitrogênio e emissão de CH_4 em ovinos Santa Inês.

2. LITERATURE REVIEW

2.1. Methanogenesis of rumen fermentation

Fermentation in the rumen is a complex process, enteric fermentation yields major SCFA (acetate, propionate and butyrate), fermentation acids, alcohols and other minor SCFA in which CH_4 and CO_2, H_2 and NH_3 gases are primary by-products of rumen fermentation. Production of CH_4 is the pathway of H_2 clearance in rumen fermentation (JANSSEN et al., 2010). Murray et al. (1976) estimated that, 89% enteric methane production is excreted through the lungs and only 11% through the rectum. Majority of enteric CH_4 production occurs in the reticulo-rumen and slight in hindgut region.

Simple and complex carbohydrates are converted into simple sugar with the help of microbial enzyme activity. In addition, simple sugars are fermented into SCFA and further several reactions occur to produce metabolic hydrogen. Conversion of metabolic hydrogen into H_2 by hydrogenase-expressing bacterial species and in presence of Archaea, H_2 is converted into CH_4 with the combined reaction. This process is summarized in the following equations as described by (HUNGATE, 1966; CZERKAWSKI, 1986; MOSS et al., 2000).

$$\text{Glucose} \rightarrow 2\ \text{Pyruvate} + 4H \quad [1]$$

(Carbohydrate metabolism)

$$\text{Pyruvate} + H_2O \rightarrow \text{Acetate} + CO_2 + 2H \quad [2]$$

$$\text{Pyruvate} + 4H \rightarrow \text{Propionate} + H_2O \quad [3]$$

$$2\ \text{Acetate} + 4H \rightarrow \text{Butyrate} + 2H_2O \quad [4]$$

$$CO2 + 8H \rightarrow CH_4 + 2H_2O\ \text{(methanogenesis)} \quad [5]$$

The end product H_2 is necessary to be removed from the rumen ecosystem, otherwise it can inhibit metabolism of rumen microorganisms. In addition, CH_4 production is produced only by anaerobic conditions by highly-specialized methanogenic bacteria that belong to the archaea domain, which are divided into five different form namely Methanosarcinales, Methanomicrobiales, Methanobacteriales, Methanococcales and Methanopyrales (QIAO et al., 2014).

Rumen is the chamber for billions numbers of bacteria, methanogens, protozoa and fungi. For instance, microbial populations of methanogenic archaea in concentrate based fed ruminants diets is about 10^7 to 10^9/g of rumen contents and in pasture based ruminants diets is around 10^9 to 10^{10} /g of rumen contents (ATTWOOD et al., 2011). In modern days, there are

many species that have been isolated with the application of molecular technologies and it confirms the considerable genetic diversity in methanogens in the rumen ecosystem.

Recently, Poulsen et al. (2013) has identified unknown methanogens that use of methyl groups and H_2 to produce CH_4. Metabolic pathway in terms of methyl groups with three reactions (Hydrogenotrophic, Methylotrophic and Aceticlastic) as follows (HILL et al., 2016).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \text{ (Hydrogenotrophic) [6]}$$

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \text{ [7]}$$

$$4CH_3OH \rightarrow 3CH4 + CO2 + 2H_2O \text{ [8]}$$

$$CH_3NH_2 + H_2 \rightarrow CH_4 + NH_3 \text{ (Methylotrophic) [9]}$$

$$CH_3COOH \rightarrow CO_2 + CH_4 \text{ (Aceticlastic: minor reaction in the rumen) [10]}$$

2.2 CH_4 mitigation strategies

There are several mitigation practices to reduce enteric CH_4 emissions. According to Hristov et al. (2013) these may broadly be grouped into three categories such as managemental, nutritional and advanced biotechnological strategies. From Sejian et al. (2011), the categories of mitigation strategies might be as described in Figure 2.1.

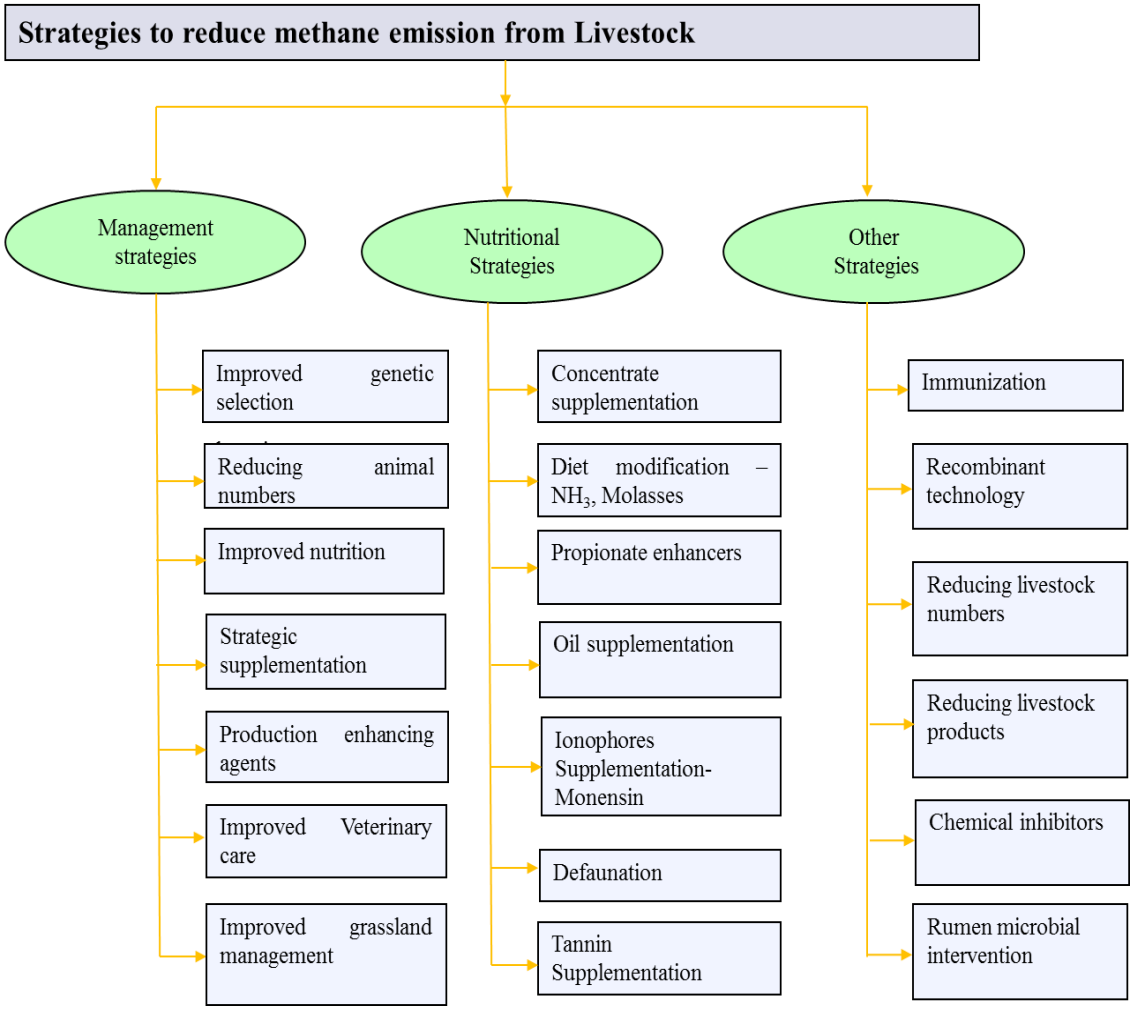

Figure 2.1. Strategies to reduce methane emission from livestock. (Source: SEJIAN et al., 2011)

Out of these strategies, the present project was conceptualized with nutritional approaches such as tannin supplementation. Tanniniferous plants and plant extracts in ruminant diets are being considered to be prominent strategies to reduce CH_4 emissions (HRISTOV et al., 2013). Briefly, the role of plant secondary compounds (PSC) such as tannin into rumen ecosystem, which has affinity towards protein to form tannin-protein complex and this complex will not be disintegrating in the rumen. Hence, protein reaches abomasum contains dietary protein and microbial protein which could be observed with lower production of NH_3-N. In other hand, tannins influence on decreasing methanogenic archaea and protozoa populations (Figure 2.2).

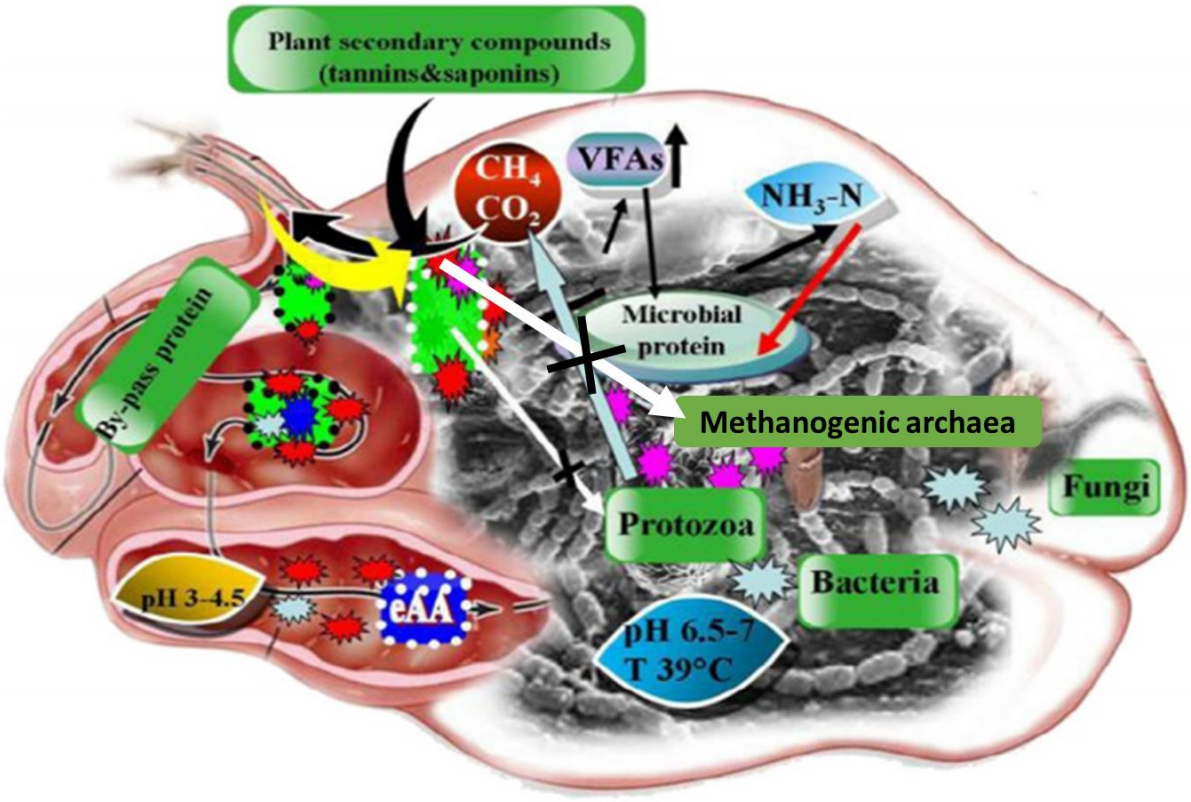

Figure 2.2. Role of PSC into rumen ecosystem. (Source & Adapted: WANAPAT et al., 2013)

2.3 Brazilian tropical plants tested for methane mitigation

Brazil has biggest biodiversity in the world. It is estimated that 20% of planet biodiversity is found in Brazil. It has considerable territorial extension composed of savanna type natural grassland, characterized by different communities of plants, shrubs, trees, and little herbaceous. There are some 43,000 to 49,000 plant species (CONVENTION ON BIOLOGICAL DIVERSITY, 2012), therefore, a few tanniferous plants were tested for methane mitigation and also replacing protein supplements in ruminants by using locally available resources (ABDALLA et al., 2012; SOLTAN et al., 2013).

2.3.1. *Leucaena leucocephala* (LL): (Bra: Leucaena)

Leucaena leucocephala (Lam.) de Wit (leucaena) is a fast growing tree and also known as the 'miracle tree' because of its worldwide invaded including in Brazil (INSTITUTO HÓRUS DE DESENVOLVIMENTO E CONSERVACÃO AMBIENTAL, 2014) Spain (DANA et al., 2003), Taiwan (CHEN et al., 2012), and Australia (WALTON, 2003). However, it is a native to Mexico and Central America. These plant species has several attributes such as highly nutritious forage tree and it can provide firewood, shade and control

in soil erosion. Botanically, LL belongs to the Mimosaceae family and it may grow upto 7-18 metres. LL leaves have also been shown as potential ruminants diets in terms of metabolic protein supply due to high protein content (SALLAM et al., 2010; SOLTAN et al., 2012) and LL has been shown anti-methanogenic properties under *in vitro* and *in vivo* studies (SOLTAN et al., 2012; 2013).

2.3.2. *Mimosa caesalpiniifolia* (MC): (Bra: Sabia)

Mimosa caesalpiniifolia Benth is a tree-sized legume and it is native to the Northeast region of Brazil. It also occurring in dry areas, where assumes shrubby (MAIA, 2004). It has the potential to be used as fodder by presenting high protein and nutrients making it an option to increase animal production especially during the dry season. It is well accepted by the animals, but may have limitations in use as forage for presenting branches with thorns, hindering the management and use of the plant (LIMA et al., 2008). Its foliage is a valuable feed source for large and small ruminants, especially during the dry due to the high nutritional value containing about 17 % crude protein (COSTA et al., 2011).

2.3.3. *Schinus molle* (SM): (Bra: Aroeira-celery)

Schinus molle is a tree which belongs to the Anacardiaceae family. The plant occurring has been reported from Minas Gerais to Rio Grande do Sul especially southern states of Brazil. In addition, it's found mainly in dry and sandy soils. It can also adapt to low fertility and stony land. It has morphological characteristics such as heights (4-8m) and leaves compound with yokes (1-12) and paniculate inflorescences terminals (LORENZI et al., 2002). Supplementation of different levels of SM can decrease gas production and organic matter degradability significantly with increasing level of supplementation, however, authors concluded that low level of SM had potential supplements to alter rumen fermentation (ONENC et al., 2013).

2.4 Tannins and their effects in rumen ecosystem

2.4.1 Tannins

Tannins are polyphenolic compounds with relatively high molecular weight and found to be in a wide range of plant species and that are commonly consumed by ruminants (MAKKAR, 2003). Tannins are classified into two groups such as condensed tannins (high molecular weight) and hydrolysable tannins (low molecular weight) and tannins have

capacity to form complexes with proteins due to presence of number of phenolic hydroxyl groups on their chemical structure (MUELLER-HARVEY, 2006).

Hydrolysable tannins (HT) are composed of polyol (glucose, glucitol, quinic acids, quercitol and shikimic acid) as a central core and it is esterified with a phenolic group. Hydrolysable tannins compound such as Gallic and Ellagic acid (PATRA; SAXENA, 2011). However, condensed tannins (CT) or proanthocyanidins tend to occurs as complex mixture of flavan-3-ol (epi) catechin and (epi) gallocatechin units with the interflavonoid linkages of C4–C8 and C4–C6 (FERREIRA et al., 1999). There are many other monomeric units (profisetinidins, probinetidins and proguibortinidins) found in condensed tannin (HASLAM, 1989). Quebracho tannins contains mainly profisetinidins (HEMINGWAY, 1989).

There are several colorimetric methods to analyse different tannins, but there are few studies to quantify tannin in their chemical structures in terms of monomeric composition or mean degree of polymerization (mDP) (GEA et al., 2011). By using this chemical degradation (thiolytic degradation method), we can be to quantify tannins upto molecular weight of 55000 daltons (GUYOT et al., 2001).

2.4.2 Tannin Binding Agents

To determine the adverse effects of tannins in browse species on rumen metabolism on *in vitro* and *in vivo* experiment by using polyethylene glycol or polyvinyl pyrrolidine (PVPP) as a tannin binding agents forming tannin-PEG or tannin PVPP complexes have been widely used (MAKKAR et al., 1995; TOLERA et al., 1997). In order to identify the most promising tannin complexing agents were investigated on the binding efficiency of PEG (molecular weight 2000-35,000) and PVPP (molecular weight 10,000, 40,000 and 3, 60,000) (MAKKAR, 2003; BESHARATI; TAGHIZADEH, 2011). PEG 6000 may be preferred for tannins inactivation in feedstuffs as its binding to tannins was highest at near neutral pH values (MAKKAR, 2003).

Bueno et al. (2008) reported that increase in gas production with and without binding agents such as PEG/PVPP on *in vitro* gas production technique. Results suggested that addition of PEG is more stable than PVPP complex with tannins. In contrast, Besharati and Taghizadeh (2011) showed that the addition of both PEG and PVP inactivated tannins effects and increased gas production, metabolizeable energy, and SCFA in tannin-rich diets for ruminants. Soltan et al. (2013) demonstrated that adding PEG to leucaena diet, improved DM intake, nutrient digestibility and shift in increased excretion of urine nitrogen into faecal nitrogen. Similarly, addition of PEG plus alfalfa with grape by product compared to the

control diets, resulted on enhanceing protein digestibility, microbial protein synthesis and ruminal parameters (ABARGUUEI et al., 2010).

2.4.3 Effect of tannins on CH_4 production

Tanninferous plants as feed supplement shows the most potential mitigation options with 20% reduction of CH_4 emissions (ZHOU et al., 2011; STAERF et al., 2012; HRISTOV et al., 2013). However, some tannins may be ineffective for anti-methangenic effect due to structure, molecular weight and concentration of the tannin. For example, Bhatta et al. (2009) reported that certain plants containing both HT and CT were higher potential as CH_4 reducers than those plants containing only HT.

There are three types of mechanism of action proposed on tannin against methanogensis process (TAVENDALE et al., 2005; HESS et al., 2003), they are: (i) direct effect on rumen microbes, (ii) indirect effect to decrease hydrogen production in terms of fiber digestion and (iii) inhibitory effects of tannin on rumen methanogensis with protozoa associated CH_4 production. In addition, Goel and Makkar (2012) review that HT showed inhibition of methanogens or hydrogen producing microbes (i.e direct effect) and CT had decrease CH_4 production in terms of fiber digestion (i.e indirect effect). Several studies have been confirmed to decrease CH_4 emissions by *in vitro* and *in vivo* experiment using tanniferous plant and extracts (HRISTOV et al., 2013).

Patra et al. (2006) reported that addition of methanol extract of *T. chebula* plant reduced methane emission. *Populus deltoides* leaf extracts were subjected into *in vitro* experiment showed that decrease in methane production with highest dosage (PATRA et al., 2008). Furthermore, Kim et al. (2013) found that, pine needles and gingko leaf extracts appears to have antimethanogenic properties by decreasing protozoa species. On the other hand, Patra et al. (2006) reported that, *Populus deltoides* leaves inhibited methanogenesis process without adversely affecting other rumen characteristics. Similarly, reduction of CH_4 production ($P<0.05$) in plants such as Leucaena (88%) and Acacia (89%) in which were subjected into *in vitro* gas production technique (SALLAM et al., 2010). The author attributes the methane reduction was due to direct effect on methanogens and indirect inhibitory effects on reduced H_2 production due to OM degradability. *Psidium guajava* leaves showed promising effects on antimethanogenic properties and also coupled with defaunting property due to tannin content (CHATTERJEE et al., 2014). Abdalla et al. (2012) reported that the *in vitro* effect of *Mimosa caesepiniifolia* plant showed lowest CH_4 production with relation to the highest CT content among other 9 tannin rich plants studied.

Subsequently, Abdalla et al. (2012) performed *in vivo* experiment by supplementing *Mimosa caesepineapholia* into the basal diet (corn grain; soyabean meal; cotton seed meal and Tifton hay) fed to Santa Ines sheep and the authors showed that the tannin of the plant was related with reduction of CH_4 and positive effects on organic matter digestibility. Soltan et al. (2013) reported that effects of *Leucaena leucaephala* with Santa Ines sheep were on reduction of CH_4 emissions. Recently, Rira et al. (2015) found that lower CH_4 production were induced with the supplementation of *Acacia cyanophylla*, a tanniferous plant showing high CT content (631 g/kg DM). Tan et al. (2011) found that low levels of CT extracted from LL could manipulate rumen fermentation in CH_4 production in terms of diverting H_2 away from CH_4 formation by decreasing methanogen and protozoa populations. Newbold et al. (2005) suggested that the succinate propionate pathway to produce propionate by using propionate precursors (i.e) acrylate which can reduce CH_4 with increase in acetate, propionate and TSCFA. Hence, increase in propionate could be an alterantive pathway dor H_2 disposal in rumen.

On the other hand, there are other factors which influence inhibitory effects of tannin on rumen methanogensis with protozoa associated CH_4 production. *Psidium guajava* leaves showed promising effects on antimethanogenic properties and also coupled with defaunting property due to tannin content (CHATTERJEE et al., 2014).

2.4.4 Effect of tannins on rumen fermentation

In general, higher concentration of tannin containing plants or plant extracts supplemented to ruminant diets will reduce nutrient intake and digestibility due to palatability, slowing of digestion and development of conditioned aversion. Reduction in palatability could be due to reaction with the taste receptors (irritating astringent sensation) or reaction among the tannins and salivary muco-proteins (MCLEOD, 1974). Narjisse et al. (1995) conducted experiment on factors independent on palatability and found slow digestion when tannins were infused directly into the rumen. Similiarly, Waghorn (1996) identified negative post-prandial consequences and development of condition aversions when tannins were used in diets.

It is evident that modification of digestibility by tannin ingestion is due to changes in rumen fermentation pattern and intestine digestibility. Tannins will reduce the feed digestibility along with increase in faecal excretion of N with increased in content of dietary (FRUTOS et al., 2004). However, lower concentration of CT will enhance digestibility as well as reduce protein degradation in the rumen. For instance, the effect of fiber digestibility,

when sheep fed *L. corniculatus* containing CT (25-35g/kg DM) was not affected (WAGHORN, 1987). Likewise, when ingestion of quebracho tannins at a dosages of 7.5 g and 15 g CT/Kg DM was studied, there were observed no effect on fiber digestibility (AL-DOBAIB et al., 2009). In contrast, Animut et al. (2008) reported that, *Lespedeza strial* in goat diets (15.1% CT) reduced in 25.7% the organic matter digestibility.

In other hand, tannins have been shown significant and well known effect on reduction of protein degradation in rumen due to the affinity of tannin towards protein being greater and pH of rumen favors to form tannin-protein complex (FRUTOS et al., 2004). This complex will not be disintegrating in the rumen system and therefore protein reaches abomasum contains dietary protein and microbial protein.

Hence inclusion of tannins will benefit in terms of efficiency of microbial protein synthesis (PATRA et al., 2012). Generally, the reduction of protein degradation as observed could lower production of ammonia nitrogen (WEST et al., 1993) and also increase excretion of urea N in urine (BHATTA et al., 2000; AUFRERE et al., 2008; TIEMANN et al., 2008; GRAINGER et al., 2009), which would not have benefit environmentally and also in animal production. Al-Dobaib et al. (2009) reported that, quebracho tannin in a Lucerne diet at dosages of 10 and 20 g/Kg DM improved microbial protein synthesis, however, there was no effect at the dosages of 30 g/Kg of DM. Similiarly, *Tamarindus indica* seed husks (140 g tannins/Kg DM) increased *in vitro* microbial protein synthesis (BHATTA et al., 2001).

McSweeney et al. (2001) explained two mechanisms of tannins on celluloytic bacteria, including (1) direct inhibition the cell wall or (2) secreted enzymes responsible for reduction of availability substrate due to tannin complex with nutrients. *Fibrobacter* and *Ruminocous* are the most important bacteria in order to identify the activities of cellulotic organisms in rumen. Population of *Fibrobacter succinogenes* (strain S85) was increased in concentrations of CT between 100 and 300 µg/ml from *L. corniculates*, but it was suppressed at 400 µg/ml (BAE et al., 1993). Abdalla et al. (2012) reported that nine different tested tanniniferous plants on *in vitro* studies showed that the decrease of fiber degrading bacteria (*Fibrobacter sucginogenes* and *Ruminococus albus*) was 48% and 88% compared to the Tifton.

As for the methanogenic populations, Abdalla et al. (2012) found that *Mimosa caesalpiniifolia* and LL significantly increased the ruminal population of these microorganisms by 151% and 63% respectively.

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3. Effect of native tropical plant species on *in vitro* rumen fermentation and methane emission

Abstract

The present study was designed to determine the effects of three tropical tannin plants such as *Leucaena leucocephala* (LL), *Mimosa caesalpineafolia* (MC), *Schinus molle* (SM) and one non-tannin plant *Medicago sativa* (MS) for their anti-methanogenic properties when used with and without polyethylene glycol (PEG). Efforts were also made to establish the nutrient degradability and short chain fatty acid production of these plants. As per the experimental design, the effect of three treatments such as plants, PEG and interaction between plants and PEG (P*PEG) were observed on the parameters studied. *In vitro* gas production (GP) assay was carried out using semi-automatic pressure transducer. Amongst the plants, MC had higher nutrient content, except total phenol (TP) and total tannin (TT) compared to other plants. CH_4 production in terms of mL/g OMD of tannins containing plants had significant reduction compared to non-tannin plants. Plants effects were significantly ($P<0.05$) influenced on truly degraded organic matter (TDOM) and degraded neutral detergent fiber (DNDF), especially LL had most influence on these parameters compared to other tannin containing plants. However, polyethylene glycol (PEG) and interaction between plants and polyethylene glycol P* PEG did not influence any effects on these parameters. But unlike degradability, both PEG and P*PEG significantly ($P<0.05$) influenced partitioning factor whereas plants did not influence partition factor. Tannin bioassay (%) of GP, propionate and partitioning factor had increasing trend with increase in tannin content of plant species. However, all plants when incubated in the presence of PEG did not influence rumen pH, ammonia nitrogen (NH_3-N), protozoa and total short chain fatty acid (TSCFA). Further, plants ($P<0.05$) and P*PEG ($P<0.10$) significantly influenced propionate production. However, none of the treatments influenced TSCFA including C2/C3 ratio. It was concluded that LL had positive response on antimethanogenic effects and nutrient degradability was higher than that of other tannin containing plants.

Keywords: CH_4 mitigation. Tanniniferous plants. Polyethylene glycol. Tannin bioassay. *In vitro* gas production.

3.1. Introduction

Livestock production is a major source of greenhouse gases (GHG) emissions and its contribution towards of global anthropogenic GHG emissions is about 18% (HRISTOV et al., 2013). Enteric fermentation by ruminants, manure management and rice cultivation plus fuel burn of agricultural residues were accountable for 73%, 3% and 2% of total methane emitted in Brazil respectively (CERRI et al., 2009). Land use changes (19%) and industrial processes (3%) also accounted for the total 340 Mt CO_2 eq of Brazilian emissions (ABDALLA et al., 2012).

There are many practices used to reduce enteric CH_4 emissions (HRISTOV et al., 2013). However, several countries are restricting the availability of some mitigation options. For example, European Union banned antibiotics use in livestock feeds due to risk to antibiotic resistance being passed to human pathogens (EUROPEAN UNION, 2003). Hence, researchers had an opportunity to exploit plants and plant secondary metabolites as natural alternatives to improve livestock productivity.

Uses of tannin containing plants have been studied and shown the promise for mitigating enteric CH_4 emissions (HRISTOV et al., 2013). Beauchemin et al. (2007) reported that, tannin has potential for reducing enteric CH_4 emission by up to 20%, but tannins are being reported as anti-nutritional (BUTLER et al., 1992) ; at lower concentration it improves animal productivity in terms of alterations in ruminal fermentation and microbial protein synthesis (BHATTA et al., 2012).

Brazil has irregularity in rainfall distribution, with periods of extensive drought, which induce low productivity of the herds under grazing. In a considerable territorial extension of the semi-arid northeastern part of Brazil is composed of savanna type natural grassland, characterized by different communities of plants, shrubs, trees, and herbaceous fodder. Among the several native Central and South America species of interest, 18 plants species were tested on previous studies (NOZELLA, 2006; VITTI et al., 2005, ABDALLA et al., 2012; SOLTAN et al., 2012), and three major tannin containing plants species, *Leucaena leucocephala* (LL), *Mimosa caesalpiniifolia* (MC) and *Schinus molle* (SM), were highlighted after tested by biological methods using *in vitro* gas production technique. Rodriguez et al. (2014) stated that, to estimate the magnitude of the tannin effects on rumen fermentation for certain plant species by *in vitro* incubation of tanniferous substrates with and without PEG is simple and effective tool, irrespective of their chemical nature.

Our objective was to determine the effects of LL, MC and SM for their antimethanogenic properties on *in vitro* rumen methane production, nutrient degradability and total short chain fatty acids production.

3.2. Material and Methods

3.2.1. Plant source and their chemical composition

Three tropical tannin plants *Leucaena leucocephala* (LL), *Mimosa caesalpiniifolia* (MC), and *Schinus molle* (SM), and one non-tannin plant *Medicago sativa* (MS - control) were selected to evaluate the potential effects on methane mitigation. These plant materials were collected at Sao Paulo State Agriculture Secretary (APTA Center South Region) in Piracicaba, SP, Brazil (latitude 22°42′30″ S, longitude 47°38′01″ W and 554 m above mean sea level). Aerial parts of plants with 0.5 cm in diameter were collected in the morning and freeze dried with liquid nitrogen. Approximately 500g of samples were ground using grinder machine and plant samples were milled into 2 mm sieve size. According to AOAC (2005), plant samples were evaluated for dry matter (DM), organic matter (OM), crude protein (CP). Neutral detergent fiber (NDF) and acid detergent fiber (ADL) were measured (VAN SOEST et al., 1991) with adaptions of Mertens et al. (2002). Samples were grounded to 0.25mm sieve and analyzed for total phenols (TP), total tannins and condensed tannins (CT) according to (MAKKAR, 2003).

3.2.2. *In vitro* gas production

Rumen fluid was obtained from six adult rumen-cannulated Santa Inês sheep (~65.0 kg), grazing tropical grass pasture and supplemented with ground maize and soybean meal (3 kg/100 kg live weight) with free access to a mineral premix and fresh water. Experimental animals were treated under the guidelines of the Internal Commission for Environmental and Ethics in Experimentation with Animals of CENA/USP.

In vitro gas production (GP) assay was carried out using semi-automatic pressure transducer and data logger (LANA/CENA-USP, Piracicaba/SP, Brazil) (BUENO et al., 2005). Dried 0.5 g of plant samples were tested to find out the tannin effect by using with and without PEG (tannin bioassay) in terms of quantification of tannin activity (MAKKAR et al., 1995). Samples were incubated in 25 ml of rumen fluid (equally mixed fraction of solid and liquid content) and 50 ml of buffered medium in total volume of 160 ml glass bottles with

head space of 85 ml. Bottles were sealed with 20 mm butyl septum stoppers and incubated for 24 h at 39°C.

Each treatment was incubated in six inocula for 24 h, including bottles for blanks, internal standard to enable adjustments among inocula and bottles containing substrate with PEG and without PEG. Head space gas pressure was measured at 4, 8, 12 and 24h. Gas production volume was calculated (SOLTAN et al., 2012). CH_4 concentrations were determined using gas chromatograph (Model 2014, Shimadzu, Tokyo, Japan) according to discriptions of Soltan et al. (2013). After termination of incubation (24 hr), bottles containing the residual, non-degraded substance were treated with neutral detergent for 4 hr, then were filtrated and finally washed with hot water and acetone.

The difference between the amounts of OM incubated and that of undegraded OM was considered as trully degraded OM (TDOM). The difference between the amounts of NDF incubated and those remaining undegraded were considered as degraded NDF (DNDF). The partitioning factor was calculated as per the ratio of TDOM [mg] and gas volume [ml/24 h] (BLUMMEL et al., 1997). Incubation liquor were collected for determining fermentation characteristics, pH was measured by using pre-calibrated pH meter, NH3-N according to Preston (1995), ruminal protozoa counts were microscopically counted according to the procedure described (KAMRA et al., 1991) and short chain fatty acid (SCFA) were determined according to (PALMQUIST; CONRAD, 1971).

Tannin bioactivity calculation: Increase (%) after PEG addition of all parameters was calculated was percent increase after addition of PEG, which was calculated, according to (JAYANEGERA et al., 2009; BHATTA et al., 2012; KONDO et al., 2014) explained briefly as follows:

Increase (%) after PEG addition = **(**Parameters with PEG addition (ml) − Parameters without PEG addition (ml)**)/** Parameters without PEG addition (ml) × 100

3.2.3. Statistical Analysis

The data were statistically analyzed using ANOVA with the general linear model procedure of SAS software (SAS, 2002). Data obtained from *in vitro* gas production, nutrient degradability and ruminal parameters were analyzed at 4x2 factorial design (4 plant species x 2 treatments with and without PEG) as independent variables using GLM procedures. Means were compared using the Tukey test and all significances were considered at $P<0.05$.

3.3. Results

3.3.1. Effects on chemical composition

The results of nutritional composition of the substrates are presented in Table 3.1. Amongst the plants, *Mimosa caesalpineafolia* had higher in plant composition (NDF, ADF, ADL, CP and CT) except TP and TT. Whereas the concentration of TP and TT varied widely, from 17.90 to 201.48 and 7.42 to 149.02 g of tannic acid /Kg DM with the lowest being for *Medicago sativa* (Control) and highest for *Schinus molle*.

Table 3.1 Nutritional composition, total phenol, total tannin and condensed tannin of different plants used as substrate

Nutritional Composition	MS	LL	MC	SM	sd*
Organic matter[a]	924	948	954	941	12.9
Neutral Detergent Fiber[a]	619	708	786.	572	95.1
Acid Detergent Fiber[a]	422	482	577	368	89.6
Acid Detergent Lignin[a]	140	228	359	188	93.8
Crude Protein[a]	179	171	183	89.2	44.5
Total Phenol[b]	17.9	77.4	128.8	201.5	77.93
Total Tannin[b]	7.42	58.53	74.21	149.02	58.56
Condensed Tannin[c]	0.65	65.80	112.42	34.22	47.57

Medicago sativa - **MS***; Leucaena leucocephala* - **LL***; Mimosa caesalpiniifolia* - **MC***; Schinus molle* - **SM** .[a] (g/ kg DM); [b](eq-g of tannic acid / kg DM); [c](eq-g of leucocyanidin /kg DM); sd*= standard deviation of three replicates per substrate.

3.3.2. Effect on total gas production, CH_4 production, nutrient degradability and partition factor

Net gas production was significantly (P<0.001) affected by plants, PEG and also P*PEG. Net gas production (mL/g DM) of all plants was increased with PEG addition except *Medicago sativa*. Net CH_4 production on the basis of DM showed significant (P<0.10) effects on P*PEG and non-significant effects on plants and PEG. In contrast, CH_4 production in terms of OMD and DNDF showed non-significant effects on P*PEG and significant on plants species and PEG effects (P<0.05). However, TDOM and DNDF were significant (P<0.05) only on plants and there was no influence on addition of PEG. Unlike degradability, partition factor was significantly (P<0.05) influenced by PEG and P*PEG, whereas plant species were non-significant (Table 3.2).

Table 3.2 Effect of tannin with PEG and without PEG on different plants as represented by *in vitro* rumen total gas production and nutrient degradability

Particulars	Net GP (mL/g DM)	Net CH_4 (mL/g DM)	Net CH_4 (mL/g OMD)	Net CH_4 (mL/g DNDF)	DMO (g/kg)	DNDF (g/kg)	PF *(mgTDOM/mL GP)*
Medicago Sativa							
(-) PEG	142.1[a]	15.9	10.9[a]	8.5 [a]	684.8[a]	531.5[a]	2.1
(+) PEG	136.7[a]	14.0	9.9[a]	7.9[a]	720.6[a]	584.7[a]	2.3
Leucana Leucocephala							
(-) PEG	119.3[b]	10.7	7.2[ab]	5.9[ab]	658.4[a]	542.8[a]	2.6
(+) PEG	130.5[b]	13.4	9.4[ab]	8.1[ab]	687.5[a]	581.7[a]	2.5
Mimosa Caesalpiniifolia							
(-) PEG	80.4[c]	7.6	3.4[b]	2.5[b]	445.2[b]	326.6[b]	2.6
(+) PEG	132.7[c]	14.6	8.2[b]	6.9[b]	554.8[b]	459.6[b]	2.0
Schinus Molle							
(-) PEG	97.6[ab]	7.2	4.4[b]	2.6[b]	619.1[ab]	373.5[b]	2.9
(+) PEG	155.0[ab]	14.1	9.2[b]	6.1[b]	590.5[ab]	408.3[b]	1.8
P-Value							
P	***	NS	**	**	**	**	NS
PEG	***	**	**	**	NS	NS	**
P*PEG	***	*	NS	NS	NS	NS	**
SEM	6.6	2.0	1.6	1.5	52.4	68.1	0.2

SEM, standard error of the mean, NS, Non significant, [abc]Means within column are significantly different for plants * ($p<0.10$) ** ($p<0.05$), *** ($p<0.001$); DM Dry Matter; OMD Organic Matter Degradability; DNDF Degraded Nutrient Detergent Fiber; PF Partioning Factor; TDOM Trully Degraded Organic Matter; GP Gas Production.

3.3.3. Tannin bioactivity

Tannin bioactivity was measured in terms of increase (%) with PEG addition for all plant species. There were significant effects on tannin activity as the increment of gas production in the presence of PEG for (MS (-3.8%), LL (9.4%), MC (65.1%), SM (58.8%) (SE = 6.6). Among all variables tested for tannin activity, only net GP, propionate and paritioning factor showed significant effect between the plants. Tannin activity in terms of increases in the presence of PEG on Net gas production ($P<0.0001$), partition factor ($P<0.05$) and propionate production ($P=0.018$) are shown on Figure 3.1. Gas production and propionate production had increasing trend for MS, LL, MC and SM. In contrast, partition factor showed decline trend.

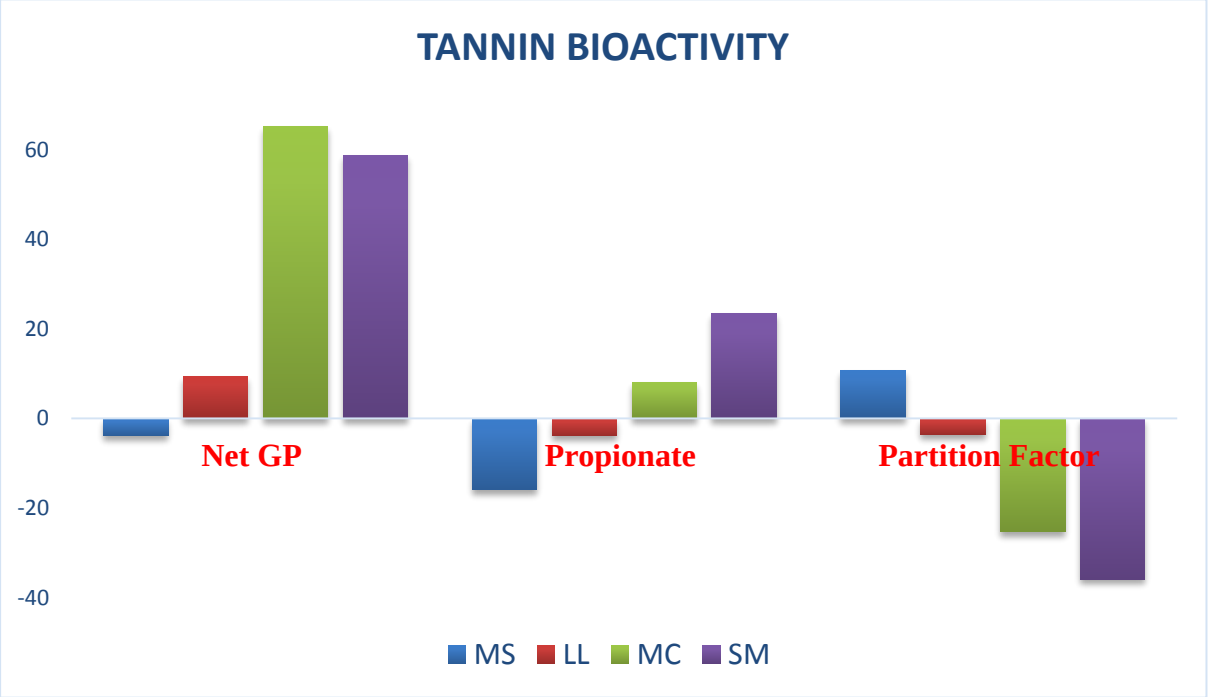

Figure 3.1 Increase of net GP, propionate,and partitioning factor with PEG addition on different plants. *Medicago sativa* - **MS**; *Leucaena leucocephala* - **LL**; *Mimosa caesalpiniifolia* - **MC**; *Schinus molle* – **SM.**

3.3.4. Effect on *in vitro* ruminal parameters

There were no significant effects of all dependent variables for pH, NH_3-N (mg/100mL), protozoa counts, short chain fatty acids (mmol/l) and the proposition of acetate:propionate (C2/C3) except for propionate production which significantly differed among the plants species ($P<0.05$) and also P*PEG ($P<0.10$), but no effects on PEG (Table 3.3).

Table 3.3 Effect of *in vitro* ruminal parameters with PEG and without PEG on different plants

Particulars	NH_3-N (mg/100mL)	PRO (N x 10^5)	C2 (mmol/L)	C3 (mmol/L)	$C4_a$ (mmol/L)	$C4_b$ (mmol/L)	$C5_a$ (mmol/L)	$C5_b$ (mmol/L)	TSCFA (mmol/L)	C2/C3
Medicago sativa										
(-) PEG	52.1	5.3	48.4	12.0^A	0.8	8.6	2.4	1.2	73.3	4.1
(+) PEG	51.3	5.4	44.5	10.1^A	0.7	7.5	2.1	1.1	65.9	4.5
Leucana Leucocephala										
(-) PEG	52.4	5.0	44.6	10.6^A	0.7	7.9	2.0	1.0	66.8	4.3
(+) PEG	54.7	2.4	43.3	10.2^A	0.7	7.7	2.2	1.1	65.2	4.3
Mimosa caesalpiniifolia										
(-) PEG	48.5	3.3	39.9	8.8^B	0.5	6.7	1.4	0.8	58.0	4.7
(+) PEG	55.0	2.9	42.2	9.5^B	0.6	7.2	2.2	1.0	62.7	4.5
Schinus molle										
(-) PEG	35.4	4.4	41.4	8.1^B	0.4	6.5	1.3	0.7	58.4	5.3
(+) PEG	49.3	3.7	45.7	10.0^B	0.6	7.5	2.0	1.0	66.8	4.6
P	NS	NS	NS	**	NS	NS	NS	NS	NS	NS
PEG	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P*PEG	NS	NS	NS	*	NS	NS	NS	NS	NS	NS
SEM	6.4	0.9	2.40	0.59	0.10	0.94	0.30	0.13	3.89	0.34

SEM, standard error of the mean, NS, Non significant, ^{ABC}Means within column are significantly different for plants; *($p<0.05$), ** ($p=0.001$), *** ($p=0.0001$), **** ($p<0.0001$); NH_3-N Ammonical Nitrogen; PRO Protoza;N Protozoa Number; C2 Acetate; C3 Propionate; $C4_a$ Iso-butyurate; $C4_b$ Butyurate; $C5_a$ Iso-Valerate; $C5_b$ Valerate; TSCFA Total short chain fatty acids; C2/C3 Acetate/propionate ratio.

3.4. Discussion

It is a constant endeavor for animal researchers to find out natural alternatives to improve livestock production and also reducing environment pollutants such as CH_4 (fermentation), P and N (manure) in ruminants (MAKKAR et al., 2009). We proposed to evaluate the effects of some tropical plant species for their anti-methanogenic properties in terms of *in vitro* rumen methane production, *in vitro* nutrient degradability and short chain fatty acids. Many studies have shown that tannin containing plants reduced methane production (JAYANEGARA et al., 2009; ABDALLA et al., 2012; SOLTAN et al., 2013; HRISTOV et al., 2013). However, there are studies which had showed negative responses (BEAUCHEMIN et al., 2007; DE OLIVEIRA et al., 2007).

3.4.1. Effects of chemical composition

In present study, we found that *Medicago sativa* (178) had higher CP content (g/kg DM) than *Leucaena leucocephala* (170), *Schinus molle* (89) and almost equal to *Mimosa caesalpiniifolia* (182); however, all three tropical tannin plants had higher NDF, TP, TT and CT content compared to *Medicago sativa* (Control). CT content of *Leucaena leucocephala* and *Mimosa caesalpiniifolia* was higher than the value reported by Abdalla et al. (2012) and lower values than reported by Soltan et al. (2013). These differences might be due to sampling site (MAKKAR; BECKER, 1998; SALEM, 2005) stage or season of plant growth (VITTI et al., 2005; SALEM, 2005).

Our selected plants were recommended to use in ruminant diets in terms of nutrient quality, considering the observations of Salem et al. (2007) which reported that high CP contents in trees and shrubs were potentially important to feed ruminants compared to low quality forages or crop by-products during dry season and also it would support ruminal degradable N supplements in semi-arid regions.

3.4.2. Effect on total gas production, CH_4 production, nutrient degradability and partitioning factor

Higher gas production and higher nutrient degradability of *Medicago sativa* suggests high fermentation than other plants and it could be due to low plant secondary compounds and also more fermentable substrates. In contrast, *Schinus molle* and *Mimosa caesalpiniifolia* had higher phenolic composition with low gas production. Similar trend were observed for some browse trees relations between CP and TP content on *in vitro* gas production (SALEM et al., 2007).

Net CH_4 production in terms of OMD and DNDF showed significant ($P<0.05$) effects of plants species and PEG addition, especially all three plants contained appreciable amount of tannin showed CH_4 reduction than those of non tannin plant (*Medicago sativa*), but there was no significants effects on interaction between plants and PEG. However, three types of mechanism of action on tannin against methanogensis have been proposed (TAVENDALE et al., 2005; HESS et al., 2003). They are: (i) direct effect on rumen microbes, (ii) indirect effect to decrease hydrogen production in terms of fiber digestion and (iii) inhibitory effects of tannin on rumen methanogensis with protozoa associated CH_4 production. Based on these mechanisms, *Mimosa caesalpiniifolia* and *Schinus molle* had high tannin content which indirectly reduces CH_4 through decrease H_2 production in terms of reduction in nutrient degradation. Similiarly, Animut et al. (2008) reported that, *Lespedeza strial* (15.1% CT) when added to goat diets reduced 25.7% the organic matter digestibility with reduction of CH_4 as 58% (in L/day basis). However, LL had tendency to reduce methane without adverse effects on nutrient degradability and it may be due to optimum amount of CT present in LL had direct effects on rumen microbes. Few studies reported that, condensed tannins had direct effects on methanogens by strongly attached with microbial enzymes, located at accessible sites of methanogens to stop their activity (FIELD et al., 1989; TAVENDALE et al., 2005). Our present findings were consistent with SOLTAN et al. (2013) without noticeable adverse effects on ruminal nutrient degradability with decrease in CH_4 emission on *in vitro* and *in vivo* studies by using *Leucaena leucephala* with and without PEG.

Leucaena leucephala showed higher nutrient degradability compared to the other two tanniferous plants and it may be due to CT content of LL. Min et al. (2003) reviewed that lower CT contents (20 to 45 g/kg DM) in temperate legumes improve ruminal fermenation including utilization of nitrogen and milk production. But, above 50 g/kg DM reduce feed intake and nutrient digestibility. In our present study, LL contained 58.5 eq-g. of tannic acid/ kg DM showed positive effects on nutrient degradability. Morever, many authors attributes that tannin effects not only depends on the amount present but also many factors such as molecular weight, size and number of site of linkages (MUELLER-HARVEY, 2006; LONGO et al., 2012). Partitioning factor of our present study was found to be significant effects on interaction beween plant and PEG, especially effect of tannin (-PEG) provides nitrogen and energy supply for the microbes utilization and it found to be more favourable for microbial growth. Similar results were obtained, when tropical grass Pennisetum purpureum and mixtures with browse legumes on *in vitro* fermentation (RODRIGUEZ et al., 2010).

3.4.3. Effects of tannin bioactivity

Tannin bioactivity is an indicator of microbial fermentation of substrates with and without PEG in terms of deactivating tannins. Many studies have proved that the activity of the tannins present in the substrates upon the fermentation parameters can be measured in terms of increases with the addition of PEG (BUENO et al., 2008; VITTI et al., 2005; RODRIGUES et al., 2014; BUENO et al., 2015). As per calulation, Gas production (after addition of PEG) had increased on *Mimosa caesalpiniifolia* showed high fermentation effect, which also contain highest CT content (112.42g/kg DM) than those of *medicago sativa*. Similar to our present findings, Bhatta et al. (2012) reported that tannin bioactivity was higher in *Ficus bengalensis* species on *in vitro* fermentation, which also contains high CT content (260g/kg DM). Furthur, increment percentage of propionate production for (*Medicago sativa>Leucaena leucocephala>Mimosa caesalpiniifolia > Schinus molle*) had increased and it is due to presence of tannin content with increase in following order. Hence, it confirms that tannin content might be responsible for shift in propionate production. In addition, *Schimus molle* had high tannin content and more shift in propionate production. Similar to our present study, Guerrero et al. (2012) reported that, 117% and 105% *C. parvifolia and A shaffneri* had propionate production with PEG. In contrast, tannin bioactivity on partition factor showed decline trend on tannin containing plants compared to the non tannin plants may be attributed to increase in gas production and decrease in efficiency of microbial protein synthesis (MAKKAR et al., 1999) in agreements with findings were reported (MLAMBO et al., 2009). However, the present findings of tannin bioactivity on GP, propionate production and PF clearly demostrated the correlation between tannin content and addition of PEG 6000.

3.4.4. Effect on *in vitro* ruminal parameters

Rumen parameters such as pH, total short chain fatty acids, NH_3-N, and protozoa counts were showed non-significant effects on with and without PEG for all plant species. Ruminal pH of all treatments varied between 6.90 to 7.00. these values were optimum pH for rumen fermenation, rumen microbial growth and their activity (WANAPAT; PIMPA, 1999; NIKKHAH et al., 2010). This result indicates that addition of PEG does not affect the rumen fermentation (TIEMANN et al., 2008). The present study of SCFA indicates the tannin effects by non tannin plants had higer production of SCFA than those of tannin plants. However, LL of tannin containing plant had numerically higher values in SCFA production. These reason is may be due to tannin plants had methane reduction was primarly due to lowered nutrient degradabily, while it confirmed through recent findings of (BHATTA et al., 2013) showed

similar trend on some plants species tested with and without PEG for protozoa population on *in vitro* studies. Furthermore, there was decrease of NH_3-N level by increasing level of tannin content present in plants. However, structure of tannin could be a reason behind lowering NH_3-N and Bhatta et al. (2013) described that samples containing condensed tannin will be responsible for lowered NH_3-N than those of hydrolyzable tannin containing plants. However, there was increasing trend of NH_3-N with PEG could be due to higher CP degradability (GETACHEW et al., 2000) or utilization of N can be improved (SALEM et al., 2007) and another possibility that absence of tannin overcoming the inhibition of microbial deaminase (LEINMULLER; MENKE, 1990). Among ruminal parmeters tested, there was significantly decrease of propionate production in MC and SM compared with LL and MS. It could be due to total tannins and phenolic content, which depress nutrient degradability and also lower the level of gas production. Similiarly, Hassen et al. (2016) reported that high and medium level of tannins decrease individual molar proportions of SCFA and total SCFA. Protozoa count number was non-significant among the treatments. Hence, our study was consistent with JAYANEGARA et al. (2012) reported by meta-anlysis that protozoa counts had no direct relationship with dietary tannin.

3.5. Conclusions

Our findings of present study, *Leucaena leucocephala* had positive response on antimethanogenic effects as well as the nutrient degradability showed higher than that of other studied tannin containing plants. Further studies are needed to be conducted with selected plants for metabolomics approach to find individual bioactive compound rather than group of compounds against anti-methanogenic activity. However, we anticipate that advances technology like GC-MS will provide unprecedented data on the distribution of component existing in plant extracts.

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4. Isolation and characterization of chemical components of *Leucaena leucocephala* for anti-methanogenic properties by using gas chromatography and mass spectroscopy (GC-MS)

Abstract

Studies emphasized that group of plant secondary metabolites (PSM) like saponins, flavonoids and tannins had the ability to manipulate rumen fermentation positively, simultaneously reducing CH_4 formation. The aim of the research was to find out individual bioactive compound with anti-methanogenic activity. *Leucaena leucocephala* plant samples were extracted with methanol and ultra sonication. Crude methanolic Leucaena extract (CMLE) (8.58 g) was further extracted with different organic solvents having increasing polarity resulted i.d. extracts of hexane (1.03 g), chloroform (0.34 g), ethyl acetate (0.48 g), butanol (0.77 g) and residual crude fractions (1.99 g) respectively. Assessment of phyto-constituents in such organic extracts was subjected to find out individual bioactive compound with modified GC–MS method. There were identified 35 components from the chromatograms of the different organic solvent extracts. Further, dried 0.5 g of ground alfafa (*Medicago sativa*) (positive control) and individual solvent extracted from crude methanolic extracts at three different levels (125 µg/mL, 250 µg/mL and 500 µg/mL) were tested for anti-methanogenic properties in terms of *in vitro* gas production and nutrient degradability. Major abundant compounds present at the relative percentages of methanolic hexane extracts (MHE) was found to be stigmasterol trimethyl ester (TMS) (12.6 %), neophytadiene (4.4 %), palmitic acid TMS (6.3 %), α-Linolenic acid TMS (10.4%) and 2, 3, 5, 6-tetra methyl anisylbenzene (8.2 %). Similiarly, methanolic chloroform extracts (MCE) contains stigmasterol trimethyl ester (TMS) (7.04 %), neophytadiene (2.33 %), palmitic acid TMS (6.46 %), α-Linolenic acid TMS (8.66 %) and dimethyl-pentadecyl-amine (5.89 %). The addition of different extracts in terms of nutrient degradability, true organic matter degradability (TDOM) and degradade neutral detergent fiber (DNDF) were increased by all extracts, but effects were differed. MHE, MCE, methanolic ethyl acetate extracts (MEE) and methanolic butanol extracts (MBE) exhibits linear and quadratic response except MRE had linear effects. Similarly, partitioning factor of MHE, MCE and MEE showed linear ($P<0.001$) and quadratic ($P<0.001$) effects, whereas for MBE and methanolic residue extracts (MRE), there were linearly significant to the control. The pH of the ruminal fermentation were similar widely (6.81 to 6.86) between the treatments. There was no significant effect on ruminal parameters such as NH3-N, total protozoa counts, total short chain fatty acids (TSCFA) and the proportion acetate: propionate with the addition of different plant extracts compared to the control. This study explained that hexane extract and chloroform extract from whole plant methanolic extract was effective as anti-methanogenic activity in modifying ruminal degradation of nutrients. The most active components still have to be identified by further fractionation of hexane extract.

Keywords: *Leucaena leucocephala*. Anti-methanogenic properties. GCMS.

4.1. Introduction

Livestock production is a significant source of greenhouses gas emissions and there is a constant endeavor for animal nutritionists to improve the animal performance in terms of manipulation of rumen microbial ecosystem, enhancing fibrous feed digestibility, methane reduction and excretion of nitrogen by ruminants (PATRA et al., 2006).

According to Hristov et al. (2013), nutritional strategies are needed to meet the global demand for food with a minimal impact on the environment. Feed additives are included into animal diets to improve feed quality, growth, nutrient deficiency, adsorb toxins, breakdown of anti-nutritive factors and reduce methane production in the rumen (DURMIC et al., 2014). Ionophores and antibiotics had been introduced to manipulate ruminal fermentation (RUSSELL, 1987), but, European Union had banned antibiotics use in animal feeds due to human food safety (EUROPEAN UNION, 2003). The supplement of probiotics into the rumen microorganisms, which increase propionate or butyrate and reduce the protozoa number resulted reduction in methane emissions (IQBAL et al., 2008), but, its usage in large scale production to mitigate CH_4 emissions is very expensive.

Therefore, the use of plants or plant extracts containing high level of plant secondary metabolites might improve animal performance without harshening safety issues because most of such plants do not compete for the production of human food. Several studies emphasized that group of plant secondary metabolites (e.g. saponins, flavonoids, and tannins) seems to present the ability to manipulate rumen fermentation in a favorable way, thus lessening the CH_4 formation (BHATTA et al., 2009; GOEL; MAKKAR, 2012; ABDALLA et al., 2012).

Brazil has extensive savanna type natural grassland, characterized by different communities of plants, shrubs, trees, and herbaceous fodders which present tannins in their composition and has potential as animal feed (VITTI et al., 2005a; 2005b; ABDALLA et al., 2012; SOLTAN et al., 2012; LONGO et al., 2012; 2013). Among such different plants, *Leucaena leucocephala* (LL), a leguminous plant, had shown a positive effect on anti-methanogenic activity *in vivo* (SOLTAN et al., 2013).

The purposes of this research were to find individual bioactive compound rather than group of compounds with anti-methanogenic activity through gas chromatography and mass spectroscopy (GC-MS) analysis of different organic solvent extracts with increasing polarity and *in vitro* ruminal fermentation evaluation. The literature search revealed that still no

studies has been tested *in vitro* anti-methanogenic activity and characterize the chemical constituents in different crude extracts of this plant species.

Therefore, the aim of this study was to evaluate the effect of different organic extracts from the whole plant methanolic extract of *Leucaena leucocephala* on *in vitro* gas production and characterize the chemical constituents and fingerprint by using GC-MS.

4.2. Material and Methods

Consumable parts of the leucaena plant (leaves and small stems with <1 cm diameter) were collected in the Piracicaba river shore in Piracicaba, Sao Paulo state, Brazil. Plant samples (±5 kg) were separated immediately and dried at 40°C for 48 h. Approximately 500 g of samples were ground using grinder machine and plant samples were milled into 2mm sieve size.

4.2.1. Laboratory analysis

Plant samples were analyzed for dry matter, organic matter, and crude protein using the procedure of AOAC (2000). Analysis of neutral detergent fibre (NDF) and acid detergent fibre (ADF) were processed according to VAN SOEST et al. (1991). Extractable total phenols (TP), tannins (TT) and condensed tannins (CT) were estimated and expressed as tannic acid equivalents and leucocyanidin equivalent. Total phenols were determined with Folin-Ciocalteau reagent (MAKKAR et al., 1993; MAKKAR, 2003). Extractable tannins were measured by the difference in total phenols (measured by Folin-Ciocalteau reagent) before and after treatment with insoluble polyvinyl polypyrrolidone (PVPP), as this polymer binds strongly to tannins (MAKKAR et al., 1995). Condensed tannins were analysed by the HCl-butanol method (MAKKAR, 2003).

4.2.2. Extraction method

Ground plants samples of leucaena and alfafa (50 g) were extracted with methanol solvent (500 mL) at room temperature for 60 min (20 min×3) using ultrasonic cleaner (ANNEGOWDA et al., 2013). After extraction, it was filtered through whatman filter paper no.1 to obtain particle-free crude extract. The methanol solvent was fully evaporated with the help of rotary evaporator.

Crude methanolic extract of leucaena (8.58 g) was suspended in 100 ml double distilled water. The suspension was transferred into separating funnel and extracted twice with 100 ml of different organic solvents with increasing polarity such as hexane, chloroform, ethyl acetate and butanol. All the crude extracts were filtered using filter paper (Whatman No. 41). All extracts were concentrated and evaporated using rotary evaporator and dried under vacuum.

4.2.3. GC-MS Analysis

Assessment of phyto-constituents in different organic extracts obtained from whole plant methanolic extract was subjected to find individual bioactive compound with the modified GC–MS method reported (SILVA et al., 2008). About 15 mg dried of crude alfafa methanolic extract and individual solvent extract were suspended with 200 µL of N-methyl-N trimethylsilyl trifluoroacetamide (MSTFA) in a sealed glass vial for 15 min at 60°C to form TMS derivatives. Reagents were evaporated with the aid of nitrogen gas and vials were reconstituted with 700 µL hexane and filtered before analyzing with GC-MS.

Shimadzu gas chromatography mass spectrometer (GCMS-QP2010) coupled with quadrupole detector was used for the separation of various phytoconstituents using RTX5MS capillary column (30 m×0.25 mm×0.25-µm film thickness). Oven temperature was maintained at 80°C for 6 min and then gradually increased into two times at 7°C/min to 310°C for 15 min and then at 5°C/min to 320^0 C maintained at the temperature for 5 min. Helium gas with a flow rate of 1.0 mL/min was used as a carrier gas and 1 µL of sample in split mode (1/40) was injected for the GC–MS analysis purpose. The mass spectrometer was operated with the EI ion source operating at 70 eV and acquisition range between 40 and 800 m/z, scan rate of one scan per 0.5 second. Each resolved compound was identified by Natural Institute of Standards and Technology mass spectral library version 2.0 (NIST02 Library, NIST, USA).

4.2.4. Treatments and experimental design

In vitro gas production (GP) assay was carried out (BUENO et al., 2005) using four adult rumen-cannulated Santa Inês sheep, treated under the guidelines of the Internal Commission for Environmental and Ethics in Experimentation with Animals of CENA/USP. Dried 0.5g of ground leucaena and alfalfa plant (positive control) and different crude methanolic extracts of each tested plant (hexane, chloroform, ethyl acetate, butanol and

methanol residue) at three different levels (125 µg/ml, 250 µg/ml, 500 µg/ml) were tested for antimethanogenic properties.

Samples were incubated in 25 mL of rumen fluid (equally mixed fraction of solid liquid content) and 50 ml of buffered medium in total volume of 160 ml glass bottles with head space of 85 ml (BUENO et al., 2005). Bottles were sealed with 20 mm butyl septum stoppers and incubated for 24h at 39^0C. Each treatment was incubated in four inocula with duplicate and incubated for 24 h with including bottles for blanks, internal standard (Tifton-85 sp.) to enable adjustments among inocula and bottles containing substrate with extraction. Head space gas pressure were measured at 4, 8, 12 and 24 h and gas production volume was calculated (SOLTAN et al., 2012). CH_4 concentrations were determined using gas chromatograph (Model 2014, Shimadzu, Tokyo, Japan) as described by Soltan et al. (2013).

4.2.5. *In vitro* ruminal parameters

Truly degraded organic matter (TDOM) was determined as per Blummel and Becker (1997) after 24 h incubation. Residual bags containing non-degraded substance were removed from the bottles and kept immediately on ice to stop the microbial fermentation process. Sequentially, bags were first treated for 1 h at 90°C with neutral detergent solution then with acid detergent solution and finally washed with hot water and acetone. TDOM was considered the difference between the amounts of organic matter (OM) incubated and that of undegraded OM. Similarly, the difference between the amounts of either neutral detergent fibre (NDF) or acid detergent fibre (ADF) incubated and those remaining undegraded were considered either as degraded NDF (DNDF) or degraded ADF (DADF), respectively. Partitioning factor (PF) as indicator of microbial efficiency was calculated by means of ratio between TDOM [mg] and gas volume [ml in 24 h] (BLUMMEL; BECKER, 1997).

The pH of rumen liquor was recorded immediately after termination of 24 h incubation using a pre-calibrated digital pH Meter. An aliquota of 3 mL of liquid was collected for NH_3-N concentration, measured according to Preston (1995) and ruminal protozoa counts were microscopically counted according to the procedure described by Kamra et al. (1991). Short chain fatty acids (SCFA) was determined according to Palmquist and Conrad (1971).

4.2.6. Statistical data analysis

The data were statistically analyzed using ANOVA with the general linear model procedure of SAS software (SAS, 2001). Data obtained from *in vitro* gas production, nutrient degradability and ruminal parameters were analyzed at 5 x 3 factorial design (5 different solvents x 3 different levels (125 µg/ml, 250 µg/ml and 500 µg/ml)) as independent variables using GLM procedures. Means were compared using the Tukey test and all significances were considered at $P<0.05$.

4.3. Results and Discussion

The chemical composition (NDF, ADF, ADL, CP and TP) of leucaena was found to be 708, 482, 228, 179 g/kg DM and 77.4 g of tannic acid /kg DM, respectively. Chemical composition of leaucaena obtained was lower than the value reported by Soltan et al. (2013) except TP showed higher content. Crude methanolic Leucaena extract (CMLE) (8.58g) was obtained further extracted with different organic solvents having increasing polarity resulted i.d. extracts of hexane (1.03 g), chloroform (0.34 g), ethyl acetate (0.48 g), butanol (0.77 g) and residual crude fractions (1.99 g) obtained respectively. Similiarly, characterization of chemical compounds in different crude extracts from neem leaves were reported (HOSSAIN et al., 2013).

4.3.1. Compounds identified in different extracts

The identification and characterization of individual bioactive compounds were evaluated for anti-methanogenic activity from the chromatograms of different organic solvent extracts in whole methanolic extracts by using GC-MS. The identities of different coumpounds are shown in Table 4.1. Studies have reported that LL contains many plant secondary metabolites such as flavonoids (ADEKUNLE; ADEROGBA, 2008), ficaprenol-11 (polyprenol) and squalene (SALLAM et al., 2010). However, present study identified a whole range of 35 individual components in different methanol solvent extracts (Table 4.1). Compounds were found to be similar more than 85% of the search results by comparison with data from literature and the profiles from the Wiley 138 and National Institute of Standards and Technology'98 (NIST'98) were considered.

Table 4.1 Identity of constituents determined by gas chromatography/mass spectrometry in the fractionation of methanol extracts of *leucena leucephalla* plant species

S.No	Names	RT	CMAE	MHE	MCE	MEE	MBE	MR
FLAVANOIDS								
1.	Epicatechin	34.61	-	-	-	4.48	1.09	-
2.	Quercetin	49.50	-	-	-	22.1	8.08	-
3.	Myricetin	49.74	-	-	-	-	1.29	-
4.	Kaempferol.	50.09	-	-	-	2.98	-	-
STEROIDS ALCOHOLS								
5.	2, 3, 4-Tris (oxy) butanal.	19.14	1.01	-	-	-	-	-
6.	Silane (3, beta, 22E)-stigmasta-5, 22-dien-3-yl) oxy).	38.84	-	-	-	1.07	-	-
7.	Delta, 5-cholesten-3, beta-ol-trimethyl ester.	40.33	-	1.68	-	-	-	-
8.	Stigmasterol,TMS	41.32	-	12.63	7.04	-	-	-
TERPENE								
9.	Neophytadiene.	24.14	-	4.39	2.33	-	-	-
10.	Phytol.	24.76	-	1.41	0.72	-	-	-
11.	Norolean-12-ene.	41.62	-	0.43	-	-	-	-
FATTY ACIDS								
12.	Palmitic acid, TMS	27.02	-	6.31	6.46	2.14	-	-
13.	Hexadecanoic acid, TMS	27.06	8.35	--	-	-	-	-
14.	Linolsaeure, TMS	29.18	2.89	3.70	2.48	-	-	-
15.	α-Linolenic acid, TMS	29.28	5.78	10.41	8.66	1.91	-	-
16.	Octadecanoic acid, TMS	29.56	2.24	2.12	2.15	0.74	-	-
17.	Eicosanoic acid, TMS	31.84	-	0.37	-	-	-	-
18.	Hexadecanoic acid, 2,3- Bis(trimethylsilyl) oxy)propyl ester	33.54	-	-	1.68	0.57	-	-
PHOSPHORIC ACIDS								
19.	Phosphoric acid, TMS	14.36	-	1.82	1.22	-	-	-
20.	Phosphoric acid, bis(trimethylsilyl) 2,3-bis(trimethylsilyl)oxy)propyl ester	23.31	-	0.84	0.37	-	-	-

To be continued

Continuation

CARBOXYLIC ACIDS								
21.	2-Piperidinecarboxylic acid phosphoric acid, tris TMS	16.17	-	0.25	0.57	0.83	3.42	2.46
22.	Oxy-butanedioic acid (trimethylsilyl) oxy)-bis(trimethylsilyl)ester	18.64	0.68	-	-	-	-	0.52
23.	Trimethylsilyl 2, 3, 4-tris (trimethylsilyl) oxy) butanoate.	18.8	0.48	-	-	-	-	-
24.	2-Piperidinecarboxylic acid, 1- (trimethylsilyl)-5-TMS	20.6	-	0.55	1.47	1.54	3.58	4.41
25.	Zitronnensaeure tetrakis, TMS	24.14	-	-	-	-	-	0.35
26.	Hexopyranose 1,2,3,4,6-Pentakis-o-TMS	25.5	2.93	-	0.31	0.48	1.13	-
27.	3,4,5-(Oxy) Benzoic acid, TMS	26.07	-	0.96	1.7	2.06	-	-
28.	Beta-D-Galactofuranose 1,2,3,5,6-Pentakis-o- TMS	26.4	1.2	-	-	-	-	-
OTHER COMPOUNDS								
29.	2,3,5,6-Tetra-M-Anisylbenzene	39.03	-	8.20	-	-	-	-
30.	3, 7-Dioxa-2,8-Disilanonane 2,2,8,8-Tetramethyl-5-(Trimethylsilyl)-Oxy	14.33	5.74	-	-	0.81	2.13	0.89
31.	Threitol,1, 2,3,4-Tetrakis-0-TMS	-	-	-	-	-	-	0.34
32.	Acetamide 1TBDMS 1TMS	25.07	-	-	5.89	1.6	0.42	0.35
33.	Silane, [(3,7,11,15-Tetramethyl-2-hexadecenyl)oxy TMS	28.72	-	1.32	1.30	-	-	-
34.	Trimethylsilyl 2-amino-3-(1-TMS)-1H-Indol-3-yl)propanoate	29.38	-	-	-	-	0.52	-
35.	D-Fructose 1,2,3,5,6-Pentakis-o- TMS	24.18	11.98	-	-	-	1.03	-

tR (min)a = retention time, min, CMAE-Crude methanolic alfafa extract, MHE- Methanolic Hexane extract; MCE- Methanolic Chloroform extract; MEE-Methanolic Ethyl acetate extract; MBE- Methanolic Butanol extract; MRE-Methanol residue extract. [a] Compounds were similarity more than 85% of the search results by comparison with data from literature and profiles from the Wiley 138 and National Institute of Standards and Technology'98 (NIST'98).

Out of 35 compounds found for leaucaena, phenol and flavonoids (n=4), steroids alcohols (n=4), terpene (n=4), fatty acids (n=7), phosphoric acids (n=2), carboxylic acids (n=8) and other compounds (n=8) of different groups were identified by using GC-MS. Major abundant compounds present at the relative percentages of MHE was found to be stigmasterol trimethyl ester (TMS) (12.63%), neophytadiene (4.39%), palmitic acid TMS (6.31%), α-linolenic acid TMS (10.41%) and 2,3,5,6-tetra methyl anisylbenzene (8.20%). Similarly, MCE contains stigmasterol trimehyl ester (TMS) (7.04%), neophytadiene (2.33%), palmitic acid TMS (6.46%), α-linolenic acid TMS (8.66%) and dimethyl-pentadecyl-amine (5.89%).

Stigmasterol is one of the phytosterol compounds, which is used as one type of feed additive to manipulate rumen fermentation. Xi et al. (2014) suggests that phytosterols can improve rumen metabolism and increase cellulose and protein degradation. But, authors did not study the methane emission parameters. In this study, we hypothesized that stigmasterol may be responsible for CH_4 reduction.

Neophytadiene is a component, which have antimicrobial effects that may decrease nutrient degradability (MODUPE et al., 2010). However, there was no antimicrobial effect due to the concentration of neophytadiene present in this experiment. Identified flavonoids compounds existed only in MEE and MBE. In detail, the proportions of compounds were: Epicatechin (4.48%:1.09%), Quercetin (22.1%:8.08%). Flavonoids components present in the experiment would have improved nutrient degradability and microbial biomass and there was no reduction in CH_4 emissions, probably due to the concentration level of this component in plant extract.

Finally, CMAE and MRE does not contain phenol and flavonoids, steroids alcohols and terpene compounds. The major constituents that were found in CMAE were fatty acids and carboxylic acids groups and MRE contains only carboxylic acids groups (Figure 4.1).

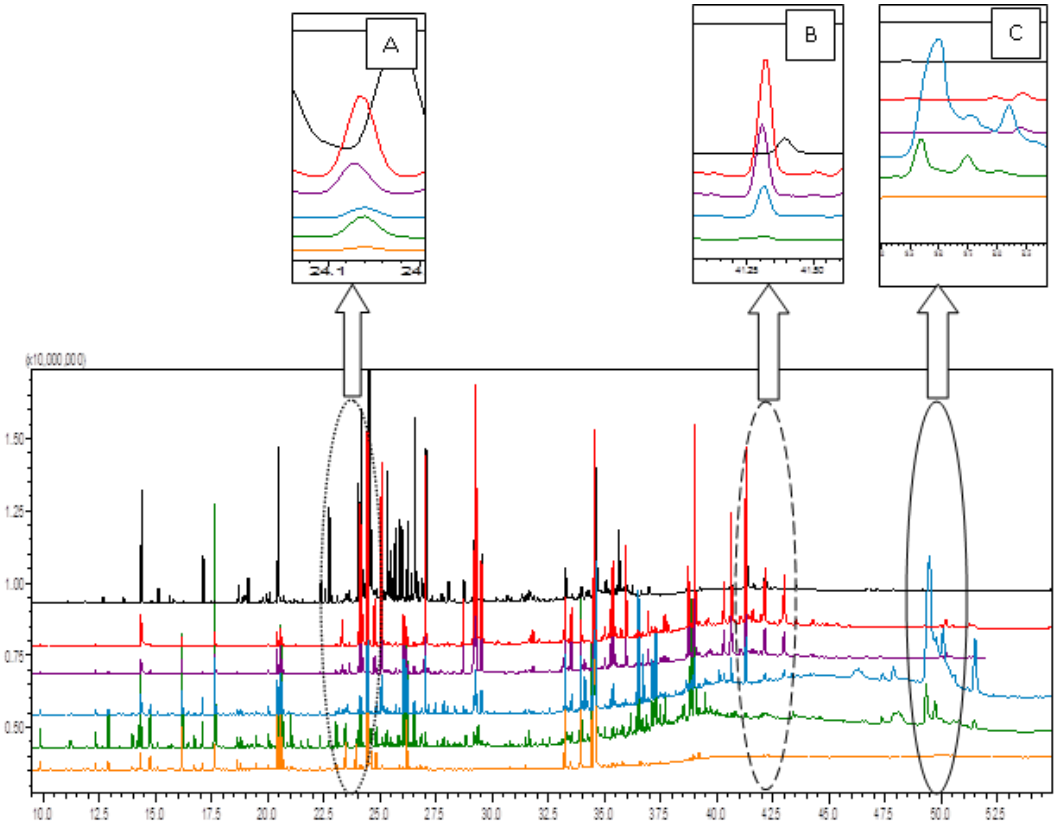

Figure 4.1 Gas chromatogram of different constituents present in fractionation of methanol extracts of *Leucaena leucocephala*. Different extract chromatogram represents Black color-Medicago Sativa Extract; Red color- Methanol Hexane Extract; Purple color- Methanol Chloroform Extract; Blue- Methanol Ethyl Acetate Extract; Green- Methanol Butanol Extract; Orange- Methanol Residue Extract; A- Neophytadiene (Terpene) Compound; B- Stigmasterol (Phytosterol) Compound; C- Flavanoids Compounds.

4.3.2. Effect on gas production

Supplementation with all extracts in different doses promoted higher gas production (mL/g OMD) ($P<0.01$) than the control groups with linear and quadratic effects. The results indicated that the all solvents and levels used in the present study did not adversely affected the rumen microorganisms. This findings supports that beneficial effects of flavonoid-rich plant extracts on *in vitro* gas production (KIM et al., 2015). However, methane production of MHE (mL/g/DM) from ruminal fermentation showed quadratic responses ($P<0.001$) up to supplementation levels of 250 µg/ml, whereas, other extracts had non-significant effects. In CH_4 production (ml /OMD) of MHE exhibiting linear effects with dose (250 µg/ml) was the most prominent doses on inhibition of CH_4 production was noted among other groups (Figure 4.2). Orthogonal polynomial contrasts were used to find out the linear (L), Quadratic (Q) and Cubic (C) response (Significant (*** $P<0.001$; ** $P<0.01$; * $P<0.05$)).

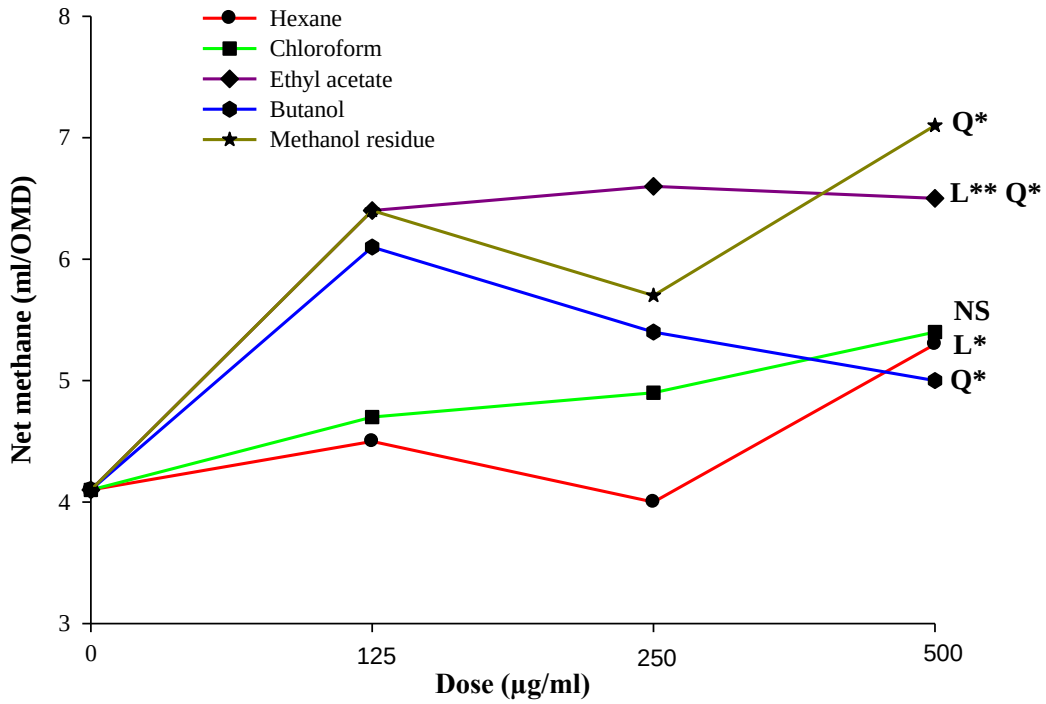

Figure 4.2 - Effects of different doses of fractionation methanol extract on *in vitro* methane production (ml/g degraded OMD), L Linear; Q Quadratic; NS Non significant

Similarly, Patra et al. (2006) reported that addition of methanol extract of *T. chebula* 95% reduced methane production with the lower dose of 0.25 ml/30 ml incubation medium and the authors attributs presence of tannins and phenolic acids might be responsible for decrease in methane production. Furthermore, Oskoueian et al. (2013) suggested that pure flavonoids compounds (myricetin, kaempferol, quercetin and catechin) was responsible for CH_4 reduction. In contrast, similar flavonoids compounds were identified in MEE and MBE in our present experiment but did not show any effects on CH_4 reduction. This is probably was due to flavonoids components extracted from plants and the low dose rate compared with pure compounds as reported against anti-methanogenesis (OSKOUEIAN et al., 2013).

In terms of CH_4 efficiency, only MHE had quadratic response compared to other extracts (Table 4.2) Non-phenol compounds can be able to reduce methane due to contain number of hydroxyl groups. Jayanegara et al. (2009) reported that higher number of hydroxyl groups are expected to reduce the methane, when tested plants contains simple phenol (benzoic, cinnamic, phenylacetic, caffeic, p-coumaric and ferulic acids) and purified tannins.

4.3.3. Effect on nutrient degradability

The addition of different extracts in terms of nutrient degradability, TDOM and DNDF was increased. However, the effects were differed with MHE, MCE, MEE and MBE exhibiting linear and quadratic responses except for MRE which had linear effects (Table 4.2). This results suggests that addition of whole methanolic extracts of LL had increased nutrient degradability without affecting ruminal ecosystem. In contrast, Patra et al. (2006) reported that, addition of different solvent extracts suppressed the IVDMD and IVOMD of feed, attributed to the detrimental effects of some secondary metabolite present in the solvent extracts.

It seems that in our current study, methanol extracts did not cause such detrimental plant secondary metabolite or otherwise plant secondary metabolites are present in very little concentration. Similarly, PF (mg TDOM ml GP^{-1}) of MHE, MCE and MEE showed linear ($P<0.001$) and quadratic ($P<0.001$) effects, whereas, MBE and MRE were showed linear significant effects to the control.

Table 4.2 Effect of different doses (µg/ml) of fractionated methanol extract of *Leucaena leucocephala* on *in vitro* rumen fermentation

Plant Extracts & Doses		Gas Production			Nutrient Degradability			Ruminal Parameters			
		GP	CH_4	CH_4E	TDOM	DNDF	PF	NH3- N	TP	TSCFA	A:P
CONTROL		52.6	10.3	7.9	402.6	108.4	1.3	49.7	10.8	63.2	5.0
MHE	125 (µg ml-1)	68.5	8.6	6.5	520.3	284.0	1.7	47.0	10.6	64.5	4.1
	250 (µg ml-1)	65.0	7.7	6.3	525.1	291.3	1.8	51.9	8.2	60.6	5.0
	500 (µg ml-1)	66.1	10.3	8.1	527.8	295.3	1.8	52.1	9.5	62.2	4.9
	SEM	3.0	0.5	0.4	21.7	32.4	0.1	2.3	1.0	4.7	0.7
	Contrast	L**Q*	Q***	Q***	L***Q*	L***Q*	L***Q*	NS	NS	NS	NS
MCE	125 (µg ml-1)	67.1	8.7	6.9	535.4	306.7	1.8	52.3	8.3	64.8	3.9
	250 (µg ml-1)	67.2	9.3	7.3	534.1	304.7	1.8	51.4	8.9	63.2	5.6
	500 (µg ml-1)	65.7	10.3	8.2	524.1	289.8	1.8	50.2	8.6	60.7	7.3
	SEM	2.7	0.8	0.6	19.3	28.9	0.1	2.8	1.0	5.3	1.3
	Contrast	L**Q**	NS	NS	L***Q***	L***Q***	L***Q***	NS	NS	NS	NS
MEE	125 (µg ml-1)	73.4	12.1	8.7	528.8	296.8	1.6	49.1	9.7	65.5	5.9
	250 (µg ml-1)	71.7	12.3	9.1	538.4	311.1	1.7	49.5	9.7	62.8	6.6
	500 (µg ml-1)	70.7	12.3	9.2	535.2	306.4	1.7	50.4	7.7	63.0	4.4
	SEM	3.6	0.9	0.4	21.1	31.4	0.1	2.7	1.2	6.2	1.2
	Contrast	L**Q**	NS	NS	L**Q**	L**Q**	L**	NS	NS	NS	NS
MBE	125 (µg ml-1)	68.6	11.6	8.8	530.6	299.4	1.8	53.1	8.6	63.2	5.1
	250 (µg ml-1)	67.8	10.4	7.8	518.3	281.1	1.7	52.9	9.2	63.8	4.6
	500 (µg ml-1)	64.2	10.0	7.7	498.2	251.1	1.7	50.4	9.0	60.3	6.5
	SEM	4.1	0.9	0.4	26.2	39.1	0.1	3.2	1.0	5.0	1.0
	Contrast	Q*	NS	NS	L**Q*	L**Q*	L**	NS	NS	NS	NS
MRE	125 (µg ml-1)	71.2	12.7	8.9	523.0	288.1	1.7	54.8	9.1	58.7	4.6
	250 (µg ml-1)	69.1	11.0	8.3	522.2	287.0	1.7	52.3	9.7	61.0	5.2
	500 (µg ml-1)	72.4	13.1	9.6	546.4	323.1	1.8	55.0	9.4	70.1	4.1
	SEM	4.2	1.5	0.8	25.2	37.6	0.1	3.6	1.1	3.8	0.7
	Contrast	L**	NS	NS	L***	L***	L**	NS	NS	NS	NS

GP- Gas Production (ml g OMD^{-1}); CH4- Methane (ml g DM^{-1}); CH4E-Methane efficiency (ml 100 GP^{-1}); TDOM- Trully Degraded Organic Matter (g kg^{-1}); DNDF-(g kg^{-1}); PF-(mgTDOM ml GP^{-1}); NH3-N (mg $100ml^{-1}$); TP-Protozoa (N x 10^5); TSCFA- Totally short chain fatty acids (mmol $mol^{-1}VFA^{-1}$); A:P- Acetate: Propionate. MHE- Methanolic Hexane Extract; MCE- Methanolic Chloroform Extract; MEE- Methanolic Ethylacetate Extract; MBE- Methanolic Butanol Extract; MRE- Methanolic Residual Extract; L Linear; Q Quadratic; C Cubic; ^{a,b,c} Means with different superscript differ (n=8). *** P<0.001; ** P<0.01; * P<0.05; ns, not Significant.

4.3.4. Effect on Ruminal Parameters

The pH of the ruminal fermentation ranged from 6.81 to 6.86 between the treatments. This indicates that our present experiment was conducted properly without any disturbance in the rumen fermentation from the added extracts; the amount of buffer was sufficient. There were no significant effects on ruminal parameters such as NH_3-N (mg 100/ml), total protozoa counts, TSCFA (mmol/mol/VFA), Acetate: Propionate with the addition of different plant extracts compared to the control (Table. 4.2). Our results suggest that higher PF value in all different solvent extracts compared to the control groups. Likewise, Salem et al. (2014) reported that influence of plant extracts with some active components result higher PF. From these above experiments, we may expect that the administration of plant extracts to ruminants will enrich the animal performance in favor of animal production and envirnomental impacts (average daily gain, SALEM et al., 2011; 2014; methane reduction, PATRA et al., 2006).

4.4. Conclusions

Our present findings suggested that chemical constituents (stigmasterol and neophytadiene) of methanol hexane extract (250 µg/ml) and methanol chloroform extract may influence on decreasing CH_4 production, increase nutrient degradability and also proportional microbial population. Furthur studies are required to explore the most active components still have to be identified by further fractionation of MHE against mechanism of action on methanogenesis.

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5 Effect of available human inedible resources *Leucaena leucocephala* to improve animal productivity and methane mitigation

Abstract

Recent days researchers are being focus on mitigation strategies and potentials that simultaneously improve animal productivity in terms of food security and livelihoods of farmers. Therefore, the objective of the present work was to study the effect of *Leucaena leucocephala* (LL) leaves, with and without supplementation of PEG, on rumen fermentation, apparent nutrient digestibility, microbial protein synthesis, nitrogen balance, rumen microbial populations and methane production in Santa Ines sheep. The animals were divided in three groups in which they were fed (i) 88% Tifton 85-hay (*Cynodon spp.*) and 12% soyabean meal (Control group, n=4); (ii) 28% Tifton 85-hay and 72% LL plus 20 ml solution containing 10 g/day/animal of PEG (With PEG group - WPEG, n=6) and (iii) 28% Tifton 85-hay and 72% LL plus 20 ml of distilled water (Without PEG group - WOPEG, n=6). Animals had free access to mineral premix and fresh water during the whole experiment. Nutrient intake (dry matter (DM), organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and crude protein (CP)) was significantly ($P<0.05$) lower in control group compared to the WPEG and WOPEG groups, except NDF intake. Nutrient apparent digestibility of DM, OM, NDF, ADF and CP did not differ among the treatments. However, daily CH_4 production of WPEG and WOPEG were significantly lower than the Control group. Furthermore, expressions of microbial populations of methanogens in WPEG and WOPEG had lower tendency than that of control. The most salient findings of this study were that, using 72% LL plant leaves in diets of small ruminants, we can get more benefits in terms of replacing the source of protein in the diet (food safety) and reduced production of CH4 (animal production).

Keywords: *In vivo*. *Leucaena leucocephala*. Anti-methanogenic properties. Food security.

5.1 Introduction

Climate change is one of the major threats on our planet with increasing population and also economical demand (SKUCE et al., 2013). According to the International Panel for Climate Change (IPCC) the rate of climate change is faster than never before in the last 1000 years and there is a possibility that a rise of average global temperatures between 1.8°C and 4.0°C within the next 90 years (YATOO et al., 2012). Hence, the impacts of global climate change are predominately threatening factors for the well-being of current and future generations (MARINO et al., 2015). Livestock sector plays a very important contribution towards to the greenhouses gas (GHG) emissions worldwide (GERBER et al., 2011). Most recent data have shown that methane (CH_4) from enteric fermentation and nitrous oxide (N_2O) from manure management generates 35% of CH_4 and 65% of N_2O of the globally contribution of the sector (7.1 billion tonnes CO_2 equivalent) (FAO, 2016).

In developing countries, animals have been an important factor in integrated livestock-crop farming systems. Animals have diversified role on production of animal protein and useful in farm manure as well as improving people livelihoods (WANAPAT et al., 2010). With expected global population growth increases around 8.3 billion people in the year 2030, it is essential to produce sufficient amount of food from locally available resources especially in developing countries. Level of consumption of animal food had increased from 10 kg/yr (1960) to 26 kg/yr (2000) and there is expected to rise up to 37 kg/yr in 2030 (WANAPAT et al., 2013; FAO, 2008; 2009). Most importantly, ruminant animals will continue as predominant factor on animal agriculture due to conversion of human inedible materials such as tree fodder, roughage, crop residue and by-products into human food. Hence, it is necessary to use locally available human inedible resources to increase animal productivity; therefore, focus must be on mitigation strategies that potentials simultaneously, improve animal productivity in terms of food security and livelihoods of farmers.

Ruminant production in tropical regions is generally based on forage grasses containing high amount of fiber and lignin content, which, at being digested, promote loss of energy when produceing enteric CH_4 and it represents loss of 8-12% gross energy (HRISTOV et al., 2013). In particular, higher CH_4 is produced when diet are based on grasses compared to the legume based diets (GOEL; MAKAR, 2012). In this context, *Leucaena leucocephala* (LL) is a leguminous shrub and it is a worldwide invaded including in Brazil (INSTITUTO HÓRUS DE DESENVOLVIMENTO E CONSERVACÃO AMBIENTAL¸

2014), Spain (DANA et al., 2003), Taiwan (CHEN et al., 2012), and Australia (WALTON, 2003).

These plant species have several attributes such as highly nutritious forage, it can provide firewood, shade and control of soil erosion. LL leaves contain high level of protein, which can be able to replace soyabean meal in ruminants diets, and also contain tannins which could reduce CH_4 production (SOLTAN et al., 2012; 2013; TAN et al., 2011). To overcome the possible restrictive effects of tannins, using polyethylene glycol (PEG) as a tannin binding agent forming tannin-PEG complexes have been used to determine the effects of tannin content in browse species on rumen metabolism (MAKKAR et al., 1995; TOLERA et al. 1997).

Several studies reported that LL can improve ruminal fermentation in terms of metabolic protein supply due to its high protein content (SALLAM et al., 2010; SOLTAN et al., 2012) and also LL have been shown antimethanogenic properties *in vitro* and *in vivo* (SOLTAN et al., 2012; 2013). In addition, compounds present in LL can improve rumen function with increase in number of microbial growth especially cellulolytic and proteolytic bacteria (HOOVER; STOKES, 1991; TAN et al., 2011). There is very few *in vivo* studies (SOLTAN et al., 2013; RODRIGUEZ et al., 2015) using LL on both rumen function and CH4 yield.

The objective of the present study was to study the effect of LL plant leaves, with and without supplementation of PEG on rumen fermentation, apparent nutrient digestibility, nitrogen balance, microbial protein synthesis, rumen microbial populations and methane production in Santa Ines sheep.

5.2 Material and Methods

The present study was conducted at the Laboratory of Animal Nutrition of Center of Nuclear Energy in Agriculture, University of São Paulo (LANA-CENA / USP), located in Piracicaba - SP, Brazil with the approval by the Institute Animal Ethical Committee (CEUA-CENA / USP).

5.2.1. Diets preparation

During rainy season, consumables parts of LL such as leaves and small stem were harvested for 2 hours between 16.00h to 18.00h at Piracicaba river shore, Piracicaba, Sao Paulo state. LL samples were allowed to dry in shade place for 96 h and dried materials were

ground through 0.5 cm screen and pooled together in big bag for storage in cool and dry place. LL plant samples (1 kg), Tifton hay (*Cynodon spp.*) and soybean meal were collected in duplicate for analysis of chemical composition (AOAC, 2005) and total phenolic content, total tannin and condensed tannin determined (MAKKAR et al., 1995; MAKKAR, 2003).

The proportion of ingredients and chemical composition of experimental diets are shown in Table 5.1.

Table 5.1 Description of the proportion of ingredients used and chemical composition of each experimental diet

Particulars	**Treatments**		
	CNL	**WPEG**	**WOPEG**
Tifton-85 (%)	88	28	28
Leucaena Leucephala (%)	0	72	72
Soybean meal (%)	12	0	0
Polyethylene Glycol (g)	0	10	0
Chemical Composition (g/kg of DM)			
Organic Matter	939.3	936.0	937.0
Crude Protein	161.0	179.3	176.5
Neutral Detergent Fiber	734.9	690.2	688.4
Acid Detergent Fiber	407.4	418.6	415.5
Condensed Tannin (%)	0.0	0.9	0.9
Gross Energy (Kcal/g)	3.90	4.01	3.99

NS, Non significant; CNL: 88% Tifton 85-hay +12% soybean meal; WPEG: 28% Tifton 85-hay + 72% LL + PEG; WOPEG: 28% Tifton 85-hay + 72% LL + distilled water.

5.2.2. Experimental Animal and housing

The present study was carried out with sixteen ewes (20 ± 1.0 kg and 6 months of age) randomly divided into three groups for a period of 28 days. Animals were adapted for 3 days for incremental diet inclusion followed by 14 days of respective diet treatments, six days for metabolism trial and five days for enteric CH_4 production assay.

The experimental diets were prepared evaluate the inclusion of LL replacing the soybean meal to provide at least 150 g/kg of CP per day to meet the nutritional requirement of growing lamb (NRC, 2007). The animals were fed within three groups: (i) Control group (CNL - n=4): 88% Tifton 85-hay (*Cynodon spp.*) and 12% soyabean meal; (ii) with PEG group (WPEG - n=6): 28% Tifton 85-hay (*Cynodon spp.*), 72% *Leucaena leucocephala* with 20 ml (10 g/day/animal of polyethylene glycol (PEG M.Wt. 6000) and (iii) without PEG

group (WOPEG - n=6): 28% Tifton 85-hay (*Cynodon spp.*), 72% LL and 20 ml of distilled water. Ewes had free access to mineral premix and fresh water during whole experiment. Animals were dewormed with albendazole at the beginning of the experiment. Ewes were housed in individual cages and reared hygienic throughout the experiment to assess the nutrient digestibility, nitrogen balance and rumen fermentation characteristics.

5.2.3. Nutrient intake, apparent digestibility and N balance

Daily offered feed, refusals, faeces and urine were collected by using metabolic cages for measuring feed intake, apparent digestibility and N balance. However, ammonia loss was prevented through daily addition of 100 ml of 10% sulfuric acid to the urine collecting flask. Suitable aliquots of faeces (10%) and urine (10%) were collected daily for 6 days and stored at -20^{0}C. After six days of metabolism trials, feed offered, refusals, faeces and urine samples were pooled for representative animals and were taken for chemical analysis.

Feed offered and refused and faecal samples were dried in a forced air oven at 50°C for 48 h and passes through a 1-mm sieve and then processed for determination of dry matter (DM), organic matter (OM), crude protein (CP), neutral and acid detergent fiber (NDF and ADF respectively) according to AOAC (2005). Nutrient intake was calculated as offered minus refused and apparent digestibility determined as nutrient intake minus excreted. Nitrogen balance was calculated as per Soltan et al. (2013) after quantification of nitrogen (N) in feed offered, refusals, faeces and urine according to AOAC (2005).

5.2.4. Rumen fluid analysis

On day 28, 4 hours after feeding, ruminal samples were collected through the oesophagus by a flexible rubber tube. Ruminal fluid was kept in pre-warmed thermos containers (39 ºC) and transport to the laboratory and analyzed immediately for pH. From these samples, 2ml of rumen fluid was collected in a tube containing 4 ml of methyl green-formalin saline solution (MFS) for protozoa counts as per the procedure of Kamra et al. (1991). NH_3-N concentration was measured by using micro-kjeldahl steam distillation according to Preston (1995).

Short-chain fatty acids were determined according to Palmquist and Conrad (1971). In brief, 2 ml of rumen fluid samples were centrifuged (11,000 rpm for 40 min at 4°C) and 800 µL of the supernatant were collected in Eppendorf tube and added 100 µL of 2-ethyl-butyric acid and 200 µL of formic acid 98 - 100 % (internal standard, MW=116.16; Sigma Chemie Gmbh, Steinheim, Germany). Again centrifuged, approximately 1.1 ml of

samples was transferred into chromatographic vial. One μL was injected onto the gas chromatograph (GC HP 5890 Series II/ integrator HP 3396 Series II/automatic injector HP 6890 Series, Agilent Technologies, Palo Alto, CA, USA). Calibration standard was prepared by known concentrations with external standards.

5.2.5. Microbial protein synthesis by purine derivatives

Estimation of microbial protein synthesis were determine after quantification of purine derivatives in urine samples by using high-performance liquid chromatography (HPLC), as adapted methodologies from Pimpa and Balcells (2002), Balcells et al. (1992) and Czauderna and Kowalczyk (2000). Collected urine samples were thawed to room temperature and homogenized for 5 mins in sonicator (ultrasound), then 5 mL of homogenize samples were taken and centrifuged for 20 mins at 1000 rpm at 4ºC. After centrifuge, 2 mL of supernatant samples were collected in a tube and added 0.25 ml of oxipurinol and 2.75 mL of ammonium phosphate monobasic (0.0025 M) following homogenization vortex. After being filtered in filter Millex ® 13 mm (0.45-μm FTFE), sample (1 mL) were transferred into 1.5 ml vials and 20 μL wer injected in the HPLC (Agilent 1100) equipped with automatic gun samples, degasser, quaternary pump, thermostat, photodiodes arrangement detectors (UV-Vis) and Zorbax ODS C18 column (250 x 4.6 mm, 5 μm particles). Standard solution was also performed for finding out the analytic curve prepared from known concentrations (500 to 1500 μM) of the studied compounds (allantoin, creatinine, uric acid, hypoxanthine and xanthine) and oxipurinol as internal standard (500 to 1500 μM). The wavelengths 225, 254, 267 and 284 nm were monitored for the quantification of compound allantoin and creatinine, hypoxanthine and xanthine, uric acid and oxipurinol respectively. Determination of microbial protein synthesis was described by Soltan et al. (2013) and amount of microbial purines absorbed from the small intestine (PD) was calculated as per equation coined by Chen and Gomes (1992) as:

$$PD_{absorbed}\ (mmol/sheep\ and\ day) = 0.84 \times PD_{excreted} + (0.385 \times BW^{0.75})$$

Conversion of total daily renal excretion of purine derivatives (PDex) to daily duodenal flux of microbial N (MN) was estimated by:

$$MN\ (g\ N/sheep\ and\ day) = PD_{absorbed}.70/\ (0.116 \times 0.83 \times 1000)$$

Where as 70=N content of purines [mg N/mmol]; 0.116 = ratio of purine-N to total N in mixed rumen microbes; 0.83 is the digestibility of microbial purines.

5.2.6. Estimation of CH_4 productions

Enteric methane emissions were measured by using sulphur hexafluoride (SF_6) tracer technique described by Johnson et al. (1994), with adaptations by Primavesi et al. (2004), Moreira et al. (2013) and Lima et al. (2014) regarding permeation tubes and yokes. Permeation tubes containing SF_6 were prepared and maintained submerged in beaker containing water at 39 °C and emission rates followed by weighing the tubes weekly for 6 weeks before the experiment. Permeation tubes with similar SF_6 emission rates were selected (16) and placed in the rumen of each ewe through the esophagus.

Rumen air sample were captured through cappilar system connected to the yokes as described Johnson et al. (1994). Yokes adapted for sheep according to Moreira et al. (2013) were fixed in individual metabolism cages and extra yokes were used to exclude ambient CH_4 interference in the measurements of CH_4. Every 24h yokes were replaced with another yokes and the gases collected by each yoke were quantified in the laboratory for measurement of CH_4 and SF6 in the gas chromatograph model GC-2010 Shimadzu™ (MOREIRA et al., 2013). Methane produced daily was determined as for Johnson et al. (1994).

5.2.7. Relative expressions of microbial populations

Frozen rumen fluid samples individually collected after 4 hours feeding as described in 5.2.4 were used for extraction of DNA using the commercial kit PowerLyzerTM PowerSoil (MoBio). The quantification of the relative abundance of total rumen bacteria, methanogenic archeas, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and anaerobic fungi was performed using specific primers of 16S rDNA (DENMAN; MCSWEENEY, 2006; DENMAN et al., 2007) through quantitative real-time PCR (ABDALLA et al., 2012) at Cellular and Molecular Biology Laboratory (CENA-USP).

DNA amplification was performed in equipment StepOnePlusTM Real-Time PCR System (Life Technologies Solution) using Syber Green ROX kit (Invitrogen), primers decribed above, pre-amplified DNA, and ultrapure water (Milli-Q). Amplification conditions were: pre-incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72° C for 30 sec, with fluorescence data collection, and at the end of the reaction, one melting curve was included the following the conditions: 95 °C for 15 sec, 60° C for 1 min and 95 °C for 15 sec. All samples were done in triplicate.

The relative size of the microbial groups methanogenic archeas (METH), *Ruminococcus flavefaciens* (RUMI), *Fibrobacter succinogenes* (FIBRO) and anaerobic fungi (FUNGI) was calculated over the reference gene 16S rDNA of total rumen bacteria (BACT)

by the equation (100 × ($2^{\Delta Ct}$) -1) according to (DEANMAN; MCSWEENEY, 2006), where delta-CT values (ΔCt) were calculated by subtracting the Ct value of the target gene (METH, RUMI, FIBRO and FUNGI), the Ct value of the reference gene (BACT).

5.2.8. Statistical Analysis

Statistical analyzes were performed using the statistical Analysis System® package (SAS Inc, NC, USA). An analysis of variance (PROC GLM) was carried out to find out the differences between treatments (CNL, WPEG and WOPEG). Means were compared by Tukey's test at a 5% probability. The experimental data were analyzed according to the statistical model:

Yi = μ + Ti + e (i), where:

Yi = value observed for characteristic analyzed;

μ = overall average;

Bi = Treatment effect I = 1, 2, 3;

e(i) = error associated with the observation (Yij).

5.3 Results

5.3.1. Nutrient intake and apparent digestibility

The effect of nutrient intake and apparent digestibility are presented in Table 5.2. There was no significant difference among treatments for sheep bodyweight. Nutrient intake (DM, OM, ADF and CP) was significantly ($P<0.05$) lower in control group compared to the WPEG and WOPEG groups except for NDF intake which were non-significant among the groups. Nutrient apparent digestibility (DM, OM, NDF, ADF and CP) did not differ among the treatments.

Table 5.2 Nutrient intake and apparent digestibility of each experimental diet

Attributes	Treatments			P-value	SEM
	Control	WPEG	WOPEG		
Bodyweight (kg)	20.1	20.0	20.9	0.591	0.65
DM intake (g/d)	613.2[b]	692.1[a]	679.7[a]	<0.001	9.53
OM intake (g/d)	563.8[b]	648.3[a]	638.2[a]	<0.001	8.68
NDF intake (g/d)	461.6	476.9	467.8	0.647	10.51
ADF intake (g/d)	233.4[b]	289.5[a]	282.1[a]	0.002	8.02
CP intake (g/d)	88.9[b]	123.4 [a]	118.8 [a]	<0.001	3.41
Apparent Nutrient Digestibility (%)					
DM	59.6	62.9	64.3	0.536	2.62
OM	61.1	65.1	66.5	0.423	2.53
NDF	60.5	67.2	63.9	0.490	3.46
ADF	56.9	58.9	56.5	0.778	2.53
CP	67.0	70.4	67.9	0.701	2.77

DM – Dry Matter; OM – Organic Matter; MM – Mineral Matter; CP – Crude Protein; NDF – Neutral Detergent Fiber. SEM: standard error of the mean; NS, Non significant; CNL: 88% Tifton 85-hay +12% soybean meal; WPEG: 28% Tifton 85-hay + 72% LL + PEG; WOPEG: 28% Tifton 85-hay + 72% LL + distilled water.

5.3.2 Nitrogen metabolism

Purine derivatives in urine (PD), microbial nitrogen supply (MN) and nitrogen balance are shown in Table 5.3. PD and MN were non-significant among the treatments. Nitrogen (N) intake of WPEG and WOPEG groups were significantly ($P<0.05$) higher than those in CNL groups, whereas excretion of faecal and urinary N were non-significant among the treatments.

Table 5.3 Determination of excreted purine derivates and estimation of microbial nitrogen absorption and the nitrogen balance in sheep fed *Leucaena leucocephala*

Attributes	Treatments			P-value	SEM
	CNL	WPEG	WOPEG		
PD (mmol/d)	6.43	6.81	5.30	0.230	0.60
PD (µmol/d/kg$^{0.75}$)	601.0	714.61	549.37	0.182	59.61
MN supply (g N/d)	2.67	2.64	2.66	0.826	0.03
N intake (g/d)	14.23[b]	19.75[a]	19.00[a]	<0.001	0.55
Faecal N (g/d)	4.71	5.79	6.06	0.106	0.38
Urinary N (g/d)	4.38	5.84	4.76	0.265	0.59
N retained (g/d)	5.14	8.11	8.18	0.096	0.89

PD-Purine derivates; MN-Microbial Nitrogen absorption; N-Nitrogen; SEM: standard error of the mean; NS, Non significant; CNL: 88% Tifton 85-hay +12% soybean meal; WPEG-With PEG: 28% Tifton 85-hay + 72% LL + PEG; WOPEG-Without PEG: 28% Tifton 85-hay + 72% LL + distilled water.

5.3.3. Ruminal Parameters

Table 5.4 show the ruminal parameters of sheep fed the experimental diets. Compared with CNL groups, ruminal pH of WOPEG group was significantly (P<0.05) higher. Ruminal ammonia nitrogen concentrations and protozoa populations showed no difference observed among the treatments. Acetate, propionate, butyrate and total short chain fatty acids (TSCFA) values were similar among groups, however, iso-butyrate, iso-valerate and the molar proportion of acetate-to-propionate of WOPEG and WPEG were significantly (P<0.05) lower compared to the CNL groups, but no difference were observed between WOPEG and WPEG. In contrast, valerate of WOPEG and WPEG was significantly increased to that of CNL groups.

Table 5.4 Effect feeding *Leuccaena leucocephala* to sheep on ruminal parameters and protozoa counts

Attributes	**Treatments**			**SEM**	**P-Value**
	CNL	**WPEG**	**WOPEG**		
pH	6.53[a]	6.71[ab]	6.76[b]	0.05	0.05
NH3-N (mg 100ml/L)	28.05	28.48	23.29	1.86	0.14
Protozoa (N x 10^5)	2.33	2.85	2.25	0.50	0.67
Total Short chain fatty acids (mmol/mol/SCFA)					
Acetate	56.07	53.98	52.96	1.61	0.49
Propionate	12.12	14.21	13.33	0.67	0.18
Iso-Butyrate	0.47[a]	0.34[ab]	0.24[b]	0.05	0.03
Butyrate	4.15	4.72	4.39	0.18	0.17
Iso-valerate	1.14[a]	0.78[ab]	0.59[b]	0.10	0.02
Valerate	0.58[b]	0.87[a]	0.72[ab]	0.05	0.01
Total SCFA	74.54	74.89	72.22	2.30	0.69
C2: C3	4.64[a]	3.82[b]	3.99[b]	0.12	<0.01

NH3-N- Ammonical Nitrogen; C2: C3- Acetate: propionate ratio; SEM: standard error of the mean; NS, Non significant; CNL: 88% Tifton 85-hay +12% soybean meal; WPEG-With PEG: 28% Tifton 85-hay + 72% LL + PEG; WOPEG-Without PEG: 28% Tifton 85-hay + 72% LL + distilled water.

5.3.4 Enteric CH_4 production

Daily enteric CH_4 production (g/kg DMI) of WPEG and WOPEG groups were significantly (P< 0.05) lower than for the CNL group and WPEG vs WOPEG were similar between these two groups (Figure 5.1).

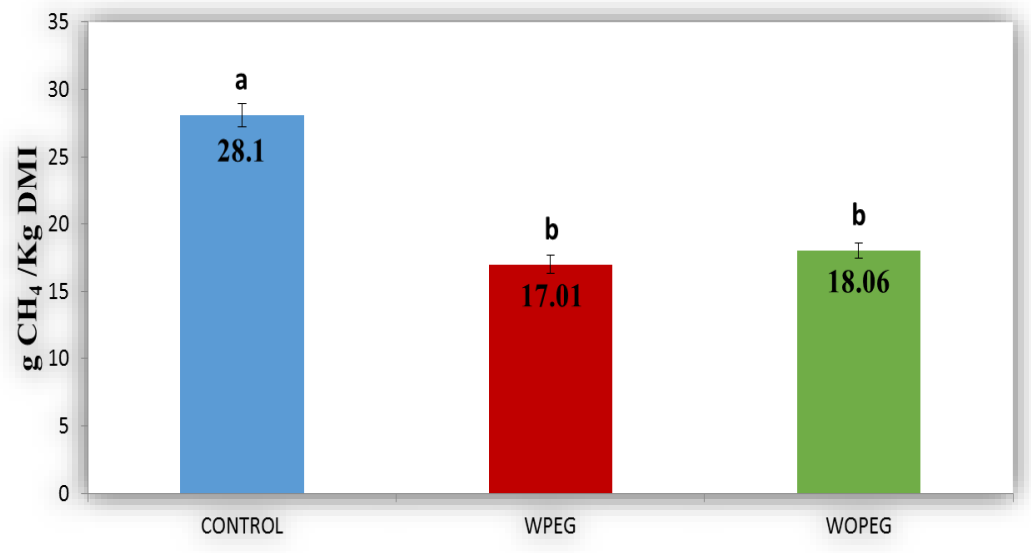

Figure 5.1 Daily enteric CH4 productions (g/KgDMI) of sheep fed experimental diets containing *Leucaena leucocephala* by using SF6 tracer technique. (CNL: 88% Tifton 85-hay +12% soybean meal; WPEG-With PEG: 28% Tifton 85-hay + 72% LL + PEG; WOPEG-Without PEG: 28% Tifton 85-hay + 72% LL + distilled water).

5.3.5 Rumen microbial populations

The ruminal microbial communities, evaluated in terms of METH, FIBRO, RUMI and FUNGI, gene expressed as proportion of total bacteria are graphically presented in Figure 5.2. FUNGI population was significantly increased in sheep fed WOPEG groups than both other groups. Expression of methanogenic archaea population was non-significant among the groups. In contrast, WPEG and WOPEG groups were increased in FIBRO population than those of CNL. But, the expression among all the groups were also non-significant. There was significant effect between WPEG and WOPEG in terms of fiber degrading bacteria RUMI and non-significant between CNL and WPEG groups (Figure 5.2).

5.4 Discussion

The principal objective of the present study was to measure the effect of LL plant leaves, with and without supplementation of PEG on rumen fermentation, apparent nutrient digestibility, nitrogen balance and methane production in Santa Ines sheep. The most salient findings of our present study were significantly ($P<0.05$) increased nutrient intake of WPEG and WPOEG than those of CNL groups. The results of nutrient intake increases may be due to palatability of LL for sheep. Similar observations were reported by Haque et al. (2008) which

showed that *Leucaena leucocephala* leaves and twigs fed goats had higher palatability than vegetative parts of maize (*Zea mays*). Soltan et al. (2013) suggested that LL leaves had better palatability compared to Tifton, maize and soyabean meal based diet.

Report shown by Hulman and Preston (1981) indicated correlation (r^2=0.98) between feed intake and level of leucaena fed to the animals. Their results suggested that there may be another reason, which might be condensed tannin (CT) content of the diet. Few studies reported that when the CT concentration exceeds 50g CT/kg, animals can reduce its feed intake may be due to acceptability and palatabiliy (WAGHORN, 2008). On other hand, feed intake increases when the level of CT was lower (WAGHORN et al., 1994).

Puchala et al. (2005) and Solaiman et al. (2010) found that forage lespedeza (*Lespedeza cuneata*) with 2.2% and 17% CT respectively, fed to goats increased DMI in relation to those fed alfalfa hay diet. The present experimental diets contained only 0.9% CT (11.9 eq. g leucocynadin/kg DM) and showed improved nutrient intake. The lack of effect on DMI between the WPEG and WOPEG groups probably was due to the low dietary CT content. This result was consistent with data shown by Soltan et al. (2013) when 35% of LL diets replaced Tifton hay for sheep.

Nutrient apparent digestibility of all three treatments groups were non-significant, which confirms there was no adverse effects on apparent nutrient digestibility. These results suggest that inclusion of LL in the experimental diet, with and without supplementation of PEG was able to provide CP digestibility similar to the soybean meal. Therefore, LL can able to provide essential nutrients, which is required for animal performance.

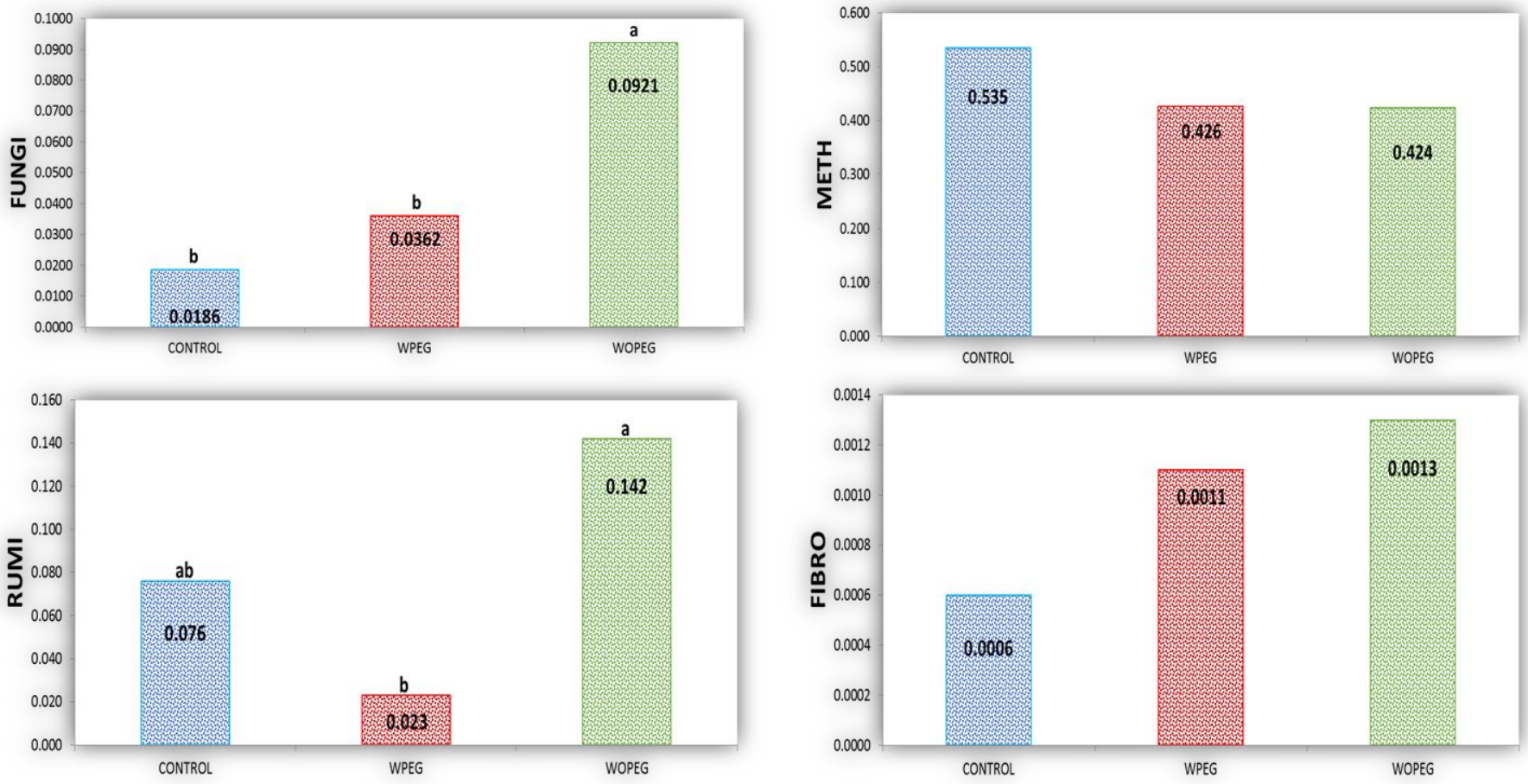

Figure 5.2 Relative abundance of methanogenic archaea (METH), fungi (FUNG), F. succinogenes (FIBRO), and R. flavefaciens (RUMI) populations expressed as proportion of total bacteria in sheep fed *Leucaena leucocephala* (CNL: 88% Tifton 85-hay +12% soybean meal; WPEG-With PEG: 28% Tifton 85-hay + 72% LL + PEG; WOPEG-Without PEG: 28% Tifton 85-hay + 72% LL + distilled water).

It is evident that modification of digestibility by tannin ingestion due to change in rumen fermentation pattern and changes in intestine digestibility but, effects of CT and PEG supplementation have been inconsistent on nutrient digestibility. CT will reduce the feed digestibility along with increase in faecal excretion of N with increasing content of dietary tannin (FRUTOS et al., 2004). However, our present study indicates that there was no negative effects of CT on apparent nutrient digestibility. Furthermore, lower concentration of CT will enhance digestibility as well as reduction of protein degradation in rumen. Phesatcha and Wanapat (2015) reported that, increase in nutrient digestibility by increasing level (upto 6 kg/head/day) of dried leucaena leaf fed to swamp buffaloes. However, in terms of fiber digestibility, all treatments were non-signifiant. Likewise, when sheep fed with *L. corniculatus* containing CT (25-35 g/kg DM) did not affect fiber digestibility (WAGHORN, 1987). In addition, ingestion of quebracho tannins (at a dosage of 7.5g and 15 g CT/kg DM) was observed no effect on fiber digestibility (AL-DOBAIB et al., 2009).

In other hand, Ben Salem et al. (2005) found that 20 g/day PEG supplementation on goat fed with kermes oak (Q*uercus cocifera. L.*) increased CP digestibility. In contrast, Yildiz et al. (2005) observed that adding 50 or 100 g PEG/kg to lambs fed *Quercus hartwissiana* (oak) leaves reduced CP digestibility significantly. In present study, there were no significant effects observed on CP apparent digestibility with supplementation of PEG.

Metabolism of nitrogen is the major process in ruminants due to protein degradation in rumen is more rapid than synthesis and production of ammonia will be absorbed through blood carried to the liver and converted into urea and excreted through urine (MCDONALD et al., 1995). Generally, estimation of purine derivatives in urine is often used to determine the microbial protein synthesis. In small intestine, dueodenal purine bases are absorbed efficiently and many metabolites are excreted via kidney with urinary recovery (PHESATCHA; WANAPAT, 2015). Firkins et al. (2007) reported that 50-80% of total absorbable protein were supplied to the small intestine by rumen microbial protein synthesis. In present study, Purine derivatives (mmol/d and µmol/d/kg0.75) and microbial nitrogen (g N/d) were non-significant among the three different groups. Few studies reported that, tannins will reduce protein degradation in rumen and therefore protein reaches abomasum contains dietary protein and microbial protein (PATRA et al., 2012). Al-Dobaib et al. (2009) reported that, quebracho tannins in a Lucerne diet at the dosage of 10 and 20g /kg DM improved microbial protein synthesis; however, there was no effect at the dosages of 30g/kg of DM. But, in present study, there was low dietary tannin content may be reason for less microbial protein synthesis and another reason could have been consumption of fermentable

nitrogen in experimental diets at higher quanity, which leads to nutritional imbalance and low effiency of microbial protein synthesis.

Furthermore, there was no significant effects on excretion of faecal and urinary N were non-significant among the treatments and also no effects on ammonia nitrogen concentrations. Eventhough nitrogen intake was higher in WPEG and WOPEG groups than CNL groups. In contrast, Soltan et al. (2013) reported that 35% Leucaena fed sheep showed a shift from urinary N to faecal N excretion and lowered NH_3-N compared to the Tifton based diet, this decrease of urinary N will benefit environmentally by through conversion of urea to ammonia in manure to nitrous oxide by utilizing ground water (ECKARD et al., 2010). In addition, supplementing legumes containing tannin decreased excretion of urinary N and NH_3-N concentrations (BEN SALEM et al., 2010; CARULLA et al., 2005). But, our results is due to low tannin dietary content and short term affects of experimental diets to the sheep.

CH_4 production (g/kg DMI) of WPEG and WOPEG was decreased by 39.5% and 35.7% compared to the CNL groups. Few studies reported that tannin will have direct effect on rumen microbes or indirect effect to decrease hydrogen production in terms of fiber digestion and inhibitory effects of tannin on rumen methanogensis with protozoa associated CH_4 production (TAVENDALE et al., 2005; HESS et al., 2003). However, our results indicate that no tannin effects were observed due to non- significant effects between WPEG and WOPEG.

Furthermore, we found significant ($p<0.01$) effects of decrease in acetate/ propionate ratio, which leads to decrease CH_4 production. Newbold et al. (2005) suggested that the succinate propionate pathway to produce propionate by using propionate precursors (i.e) acrylate which can reduce CH_4 with increase in acetate, propionate and TSCFA. Hence, increase in propionate could be an alterantive pathway for H_2 disposal in rumen. Similar to our findings, Soltan et al. (2013) reported that effects of *Leucaena leucocephala* on Santa Ines sheep showed that reduction of CH_4 emissions.

There are another factors which influence inhibitory effects on rumen methanogensis with protozoa associated CH_4 production. *Psidium guajava* leaves have showed promising effects on antimethanogenic properties and also coupled with defaunting property due to tannin content (CHATTERJEE et al., 2014). In our study, there was no significant effects on protozoa populations among the treatments. Results were consistent with (SOLTAN et al., 2013; BENCHAAR et al., 2008) reported that no effect on protozoa populations when *in vivo* experimental diets contains low level of dietary CT content.

F. succinogenes and *Ruminococcus* bacteria are most important bacteria in order to identify the activities of cellulotic organisms in rumen as well as rumen fungi also plays a role in fiber digestion. The population of FUNGI in WOPEG was increased significantly compared to other two groups. It may be due to the concentration of tannin is very optimum to increase microbial growth. In addition, it is not surprising all the fiber degrading bacteria (FUNGI, RUMI and FIBRO) in WOPEG groups. Similarly, population of FIBRO S85 increases in concentrations of CT from *L. Corniculates* between 100 and 300ug/ml, but suppressed by 400ug/ml (BAE et al., 1993). In contrast, Abdalla et al. (2012) reported that nine different tested plants on *in vitro* studies, all substrate decrease of fiber degrading bacteria of FIBRO and RUMI were 48% and 88% compared to the Tifton

There was decrease in RUMI populations in WPEG group than those other two groups, which could be due to many factors such as, PEG supplementation of other tanniferous diets fed to sheep resulted increased in ruminal volume and digesta outflow rate (BARRY et al., 1986; SILANIKOVE et al., 2001) which may reduce the population size. As for the METH populations of WPEG and WOPEG had no significant difference among the groups. Populations of METH had no influenced in reduction of CH_4 production. In contrast, Abdalla et al. (2012) found that *Mimosa caesalpiniifolia* and LL significantly increased the populations of rumen methanogens by 151% and 63% respectively. But authors attributes that increase in populations is due to the free-living methanogens.

The present research demonstrated that human inedible resources LL plant leaves contains good source of CP and also had significant decrease on CH_4 production without adverse effect of nutrient apparent digestibility and rumen methanogens populations compared to the control diets. Further studies are necessary to find out the long term effects on animal performance on production and environmental factors.

5.5 Conclusions

The most salient findings of this study were that, 72% LL plant leaves using in small ruminants diets will increase animal productivity, we can get more benefits in terms of replacing the source of protein in the diet (food safety) and reduced production of enteric CH_4 (animal production).

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6 GENERAL CONCLUSIONS

- *Leucaena leucocephala* had influenced the degraded organic matter (TDOM) and degraded neutral detergent fiber (DNDF).
- *Leucaena leucocephala* had positive response on antimethanogenic effect and its major abundant compounds present at the relative percentages of methanolic hexane extract (MHE) were found to be stigmasterol and neophytadiene which would affect enteric CH_4 production, nutrient degradability in the rumen, influencing the microbial population.
- The present research demonstrated that human inedible resources *Leucaena leucocephala* plant leaves contained good source of CP and at the low level of condensed tannin content can be able to mitigate methane without adverse effect of nutrient apparent digestibility and rumen methanogens populations.

FUTURE STUDIES

- The most active components in *Leucaena leucocephala* still have to be identified by further fractionation of MHE against mechanism of action on methanogenesis.

- To find out the long term effects on animal performance on production and environmental factors.