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AMR SALAH MORSY AMINE SELEM

Effect of propolis on ruminal fermentation, reproductive and productive performance of Santa Inês ewes

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Versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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وطنى الغالى مصر و أرواح شهداء ثورة ٢٥ يناير

Dedico essa tese para o

Meu país do coração – Egito e para todos os espiritos mortos na revolução de 25 de janeiro

I dedicate this thesis to

my country's heart Egypt and for all the dead spirits on the revolution of 25 January

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Other bees, like soldiers, armed in their stings,
Make boot upon the summer's velvet buds,
Which pillage they with merry march bring home.

Shakespeare, King Henry.

ABSTRACT

SELEM, A. S. M. **Effect of propolis on ruminal fermentation, reproductive and productive performance of Santa Inês ewes**. 2012. 119 p. Tese (Doutorado) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2012.

Propolis as natural dietary additive can be used to manipulate rumen fermentation towards less methane (CH_4) and it may affect animal reproductive and productive performance. To study the application of propolis, three studies were conducted. The first study aimed to evaluate the *in vitro* nutritive value of two types of propolis (Brazilian red propolis (BRP) and Egyptian brown propolis (EBP) for their anti-methanogenic activity, ruminal fermentation and degradability. Propolis extracts were prepared using 70% ethanol and added to a 50:50 Tifton hay to concentrate diet at three levels [0 (negative control, CTL), 25, 50 and 100 μg / 0.5 g substrate]. Each propolis were compared with monensin as positive control. Both $BRP_{50\mu g}$ and $EBP_{25\mu g}$ showed similar significant effects on CH_4 production as monensin; on average 14.2 ml/TDOM g compared with CTL (19.3 ml/TDOM). Monensin supplementation increased (P<0.001) the propionate concentration and decreased the acetate/propionate ratio, while BRP and EBP enhanced (P<0.001) the individual and total volatile fatty acids concentrations and reduced (P<0.002) protozoa count compared to CTL. The objective of the second study was to evaluate the oral administration of BRP extract to Santa Inês ewes during and after flushing period on the reproductive performance and animal health (hormonal profiles, hematological, biochemical and parasites responses). Thirty adult grazing ewes (40±2.0 kg BW) were divided into two dietary treatments, control (basal diet) and BRP (basal diet plus 3.0g of BRP/ewe/day) for 21 days. Blood and fecal samples were collected weekly for eight weeks. Oral administration of BRP did not affect (P>0.05) any of the observed reproduction traits, but there was promising improvement on the number of services per conceptions and hormones levels: increased (P<0.01) progesterone, decreased cortisol (P<0.05) and thyroxin (T_4) (P<0.01) without significant changes in triiodothyronine (T_3). Propolis resulted in increasing (P<0.01) of total leukocyte while there were no significant differences observed for other hematological parameters. Propolis increased (P<0.01) total protein and globulin but reduced (P<0.01) triglycerides, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and fecal egg counts (P<0.05) compared with control. The third study was conducted to evaluate the oral administration of BRP extract to Santa Inês ewes from 25±3 day pre-partum through 48 d post-partum on milk yield, milk composition and lamb performance. Twenty Santa Inês ewes (60 ± 2.0 kg BW) were divided into two groups: control (basal diet) and BRP (basal diet plus 3.0g of BRP/ewe/day for 21 days). Milk samples were collected weekly for seven weeks. Propolis fed group showed increasing (P<0.05) milk yield, fat content, fat yield, protein yield, lactose yield and energy corrected milk while somatic cell counts was decreased (P<0.05). Propolis increased (P<0.05) ewes body condition score. Lambs average daily gain and milk conversion ratio were improved (P<0.05) by propolis treatment. The studies highlight the potential of propolis to handle the ruminal fermentation in order to reduce the production of CH_4, as well as improved the health of ewes during the breeding season, besides increasing milk production and performance of lambs.

Keywords: Propolis. Methane. Hormones. Blood metabolites. Milk yield. Sustainability.

RESUMO

SELEM, A. S. M. **Efeito da própolis sobre a fermentação ruminal, desempenho reprodutivo e produtivo de ovelhas Santa Inês**. 2012. 119 p. Tese (Doutorado) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2012.

Própolis, aditivo natural, pode ser usada para manipular a fermentação ruminal e diminuir a produção de metano (CH_4), podendo afetar o desempenho dos animais. Foram conduzidos estudos visando avaliar a aplicação da própolis em ovinos. O primeiro experimento foi realizado com o objetivo de avaliar o valor nutritivo *in vitro* de dois tipos da própolis (Vermelho Brasileiro (PVB) e Marrom Egípcio (PME)), através da atividade anti-metanogênica, fermentação ruminal e degradabilidade. Os extratos da própolis foram preparados usando etanol e adicionados a um substrato base (50:50 feno Tifton x concentrado) em quatro concentrações [0 (controle, CTL), 25, 50 e 100 μg / 0,5 g de substrato]; sendo comparadas com a monensina como controle positivo. As própolis $PVB_{50\mu g}$ e $PME_{25\mu g}$ apresentaram redução na produção de CH_4 similar à monensina, sendo menores que o CTL. A monensina aumentou ($P < 0,001$) a concentração de proprionato e diminuiu ($P < 0,001$) a proporção de acetate / propionate, enquanto as próplis aumentaram ($P < 0,002$) as concentrações dos ácidos graxos voláteis e reduziram ($P < 0,001$) os protozoários. O segundo estudo objetivou avaliar a administração de extrato de PVB em ovelhas durante o período de "flushing nutricional" sobre o desempenho e a saúde dos animais durante a estação de reprodução. Trinta ovelhas (40 ± 2,0 kg PV) foram divididas em dois grupos, controle (dieta basal) e PVB (dieta basal com suplementação de 3,0 g de PVB / ovelha / dia) e suplementadas durante 21 dias. Amostras de sangue e fezes foram coletadas semanalmente durante oito semanas. Administração do PVB não afetou nenhuma característica reprodutiva, mas houve melhora ($P < 0.01$) no número de serviços por concepção, e aumento ($P < 0,01$) no teor de progesterona, diminuição ($P < 0,01$) nas concentrações de cortisol e tiroxina (T_4), sem efeito na concentração de tri-iodotironina (T_3). Própolis resultou apenas em aumento ($P < 0,01$) no número de leucócitos dentre os parâmetros hematológicos. A própolis aumentou ($P < 0,01$) a concentração de proteina total e de globulina, e reduziou ($P < 0,05$) os teores de triglicerídeos, transaminase oxalacética (TGO), transaminase glutamato piruvato (TGP) e contagem de ovos nas fezes quando comparado com o controle. O terceiro estudo foi conduzido para avaliar a administração do extrato de PVB para as ovelhas desde 25 ± 3 dias pré-parto até 48 dias pós-parto sobre a produção e composição do leite e desempenho dos cordeiros. Vinte ovelhas (56 ± 2,0 kg PV) foram divididas em dois grupos e suplementadas conforme descrito no segundo estudo: controle e PVB durante 21 dias. Amostras de leite foram coletadas semanalmente durante sete semanas. Próplis aumentou ($P < 0,05$) a produção de leite, conteúdo de gordura, rendimentos de gordura, proteína e lactose e leite corrigido para energia, enquanto diminuiu ($P < 0,05$) a contagem de células somáticas, mas aumentou ($P < 0,05$) a condição corporal. O ganho em peso médio diário dos cordeiros e taxa de conversão de leite foram melhoradas ($P < 0,05$) pelo tratamento com próplis. Os estudos destacam o potencial da próplis para manipular a fermentação ruminal visando redução na produção de CH_4, assim como melhorar a saúde de ovelhas durante a estação de reprodução, além de aumentar a produção de leite e desempenho dos cordeiros.

Palavras-chave: Próplis. Metano. Hormônios. Metabólitos sanguineos. Produção de leite. Sustentabilidade.

الملخص العربي

عمرو صلاح مرسى امين سليم . **تأثير البروبوليس على تخمرات الكرش و الأداء التناسلى والأنتاجى لنعاج السانتا ايناس** ٢٠١٢. ١١٩ صفحه . رسالة دكتوراه- مركز الطاقه النووي الزراعي - جامعة ساوبولو- بيراسيكابا- البرازيل ٢٠١٢.

يعتبر البروبوليس من الأضافات الغذائية الطبيعية و التى قد تستخدم لتحسين تخمرات الكرش فى المجترات و الذى بدورة يؤدى الى خفض انتاج غاز الميثان و الذى قد ينعكس بدوره على الأداء التناسلى و الأنتاجى لهذة المجترات. و لدراسة مثل هذا التاثير فقد تم عمل ثلاث تجارب مختلفة . و كان هدف الدراسة الأولى هو التقدير المعملى للقيمة الغذائية لنوعين مختلفين من البروبوليس (البروبوليس البرازيلى الأحمر و البروبوليس المصرى البنى) ضد نشاط البكتريا المنتجة للميثان و تخمرات الكرش و تحلل مادة العلف. و لقد تم استخدام المستخلص الكحلى للبروبوليس بنسبة ٧٠٪ و الذى تم اضافته الى عليقة مكونه من ٥٠ الى ٥٠ (من دريس التيفتون و المركز) حيث تم اضافة البروبوليس بواقع أربع مستويات كالأتى ((المستوى صفر و هو كان يستخدم كمجموعة مقارنة سالبة) و٢٥ و ٥٠ و ١٠٠ ميكروجرام لكل ٥٠٠ مليجرام مادة علف) و لقد تم مقارنة كل مستوى من مستويات كلا النوعين من البروبوليس بالمونانسين والذى تم اعتبارة مجموعة مقارنة موجبه . كلا النوعين من البروبوليس بتركيزات ٥٠ و٢٥ ميكروجرام للبروبوليس البرازيلى و البروبوليس المصرى على التوالى اظهرا تأثيرا معنويا =٠,٠٠٢ على خفض انتاج غاز الميثان مماثلا لتأثير المونانسين و كان مستوى الأنخفاض يقدر ب ١٤,٢ مل لكل جرام ماده عضوية حقيقية التحلل مقارنة بالمجموعة المقارنة السالبة ١٩,٣مل لكل جرام ماده عضوية حقيقية التحلل . اضافة المونانسين أدى الى زياده معنوية لتركيز حامض البروبيونات و خفض النسبة المؤيه بين حامض الاسيتك و البروبيونك , بينما كلا النوعين من البروبوليس البرازيلى و المصرى أدى الى تحسين معنويا =٠,٠٠٢ لأنتاج الاحماض الدهنية الطيارة الكلية و المنفردة و كذا أدى الى خفض عدد البروتوزوا معنويا =٠,٠٠٢مقارنة بمجموعة المقارنة السالبة . اما عن هدف اجراء التجربة الثانية فكان تقدير تأثير تجريع المستخلص الكحلى للبروبوليس البرازيلى الى نعاج السانتا ايناس خلال موسم التلقيح و الدفع الغذائى على الأداء التناسلى و الحالة الصحية العامة لهذه النعاج متضمنا (التأثير على تركيزات الهمونات المختلفة و معاير الدم الهيماتولوجية و البيلوجية و كذا الأستجابة المناعية للطفيليلت) . ثلاثون نعجة بالغة يتراوح وزنها بين ٤٠ و ٤٢ كيلوجرام تم تقسيمهم الى مجموعتان متساويتان كالأتى, المجكوعة المقارنة والتى كانت تتغذى على العليفة الأساسية فقط بدون اى معاملة و مجموعة المعامله و التى كانت تتغذى على العليقة الأساسية بالأضافة الى التجريع اليومى للبروبوليس البرازيلى بواقع ٣ جرام للنعجة فى اليوم لمده ٢١ يوم . لقد تم تجميع عينات الدم و الروث اسبوعيا ولمدة ثمانيه أسابيع متتالية و كانت أهم النتائج كالأتى, التجريع بالبروبوليس البرازيلى لم يؤثر معنويا على أى من مقايس التناسل موضوع الدراسة الأ أنه قد ظهر تحسن ملحوظ ولكن غير معنوى على عدد التلقيحات اللأزمة للأخصاب , المعاملة بالبروبوليس البرازيلى أدى الى زيادة معنوية =٠,٠٠٢فى تركيز هرمون البروجسترون و خفض معنوى =٠,٠٠٥١ لتركيز هرمونى الكورتيزول و الثيروكسين ولم يؤثر معنويا على تركيز هرمون الثيرونين ثلاثى اليود. أدى المعاملة بالبروبوليس البرازيلى الى زيادة معنويه =٠,٠٠٢ فى عدد كرات الدم البيضاء بينما لم يؤثر على أى من المعاير الهيماتولوجية الأخرى. البروبوليس أدى الى زياده معنوية =٠,٠٠٢ لتركيز البروتين الكلى و الجلوبيولين بينما انخفض معنويا =٠,٠٠٥١ تركيزات كل من الجلسريدات الثلاثية و انزيمى الكبد وكذا عدد البويضات النيماتود فى الروث مقارنة بالمجموعة المقارنة .أما عن التجربة الثالثة فكان هدف هذة التجربة هو دراسة تأثير التجريع للمستخلص الكحلى للبروبوليس البرازيلى لنعاج السانتا ايناس العشار فى الفترة الأخيرة من الحمل على انتاج و تركيب اللبن و كذا اداء الحملان المولوده من هذة النعاج .عشرون نعجة سانتا ايناس تزن فى المتوسط ٦٠ كيلوجرام تم تقسيمهم الى مجموعتين متساويتان , المجموعة الأولى وهى مجموعه المقارنة و كانت تغذى على العليقة الأساسية بينما مجموعة المعامله فكانت تغذى على العليقة الأساسية بالأضافة الى ٣ جرام من المستخلص الكحلى للبروبوليس البرازيلى للنعجة فى اليوم لمدة ٢١ يوم . لقد تم تجميع عينات اللبن اسبوعيا و لمدة سبعة أسابيع متتالية و كانت النتائج كالأتى, المعاملة بالبروبوليس البرازيلى أدى الى زيادة معنوية =٠,٠٠٥١ فى انتاج اللبن و تركيز الدهن و انتاج الدهن و البروتين و سكر اللاكتوز و محتوى اللبن من الطاقة بينما أدى المعاملة بالبروبوليس الى خفض معنوى=٠,٠٠٥١ للزيادة اليومية لوزن الحملان و كذا معدل تحول اللبن اى وزن جسم .من خلال ما تم عرضه من نتائج يمكن أن نستخلص أنه استخدام البروبوليس يؤدى الى تعديل فى بيئه الكرش للمجترات مما يؤدى الى انتاج غاز ميثان أقل و الذى بدورة ينعكس على اداء النعاج التناسلى و الأنتاجى و كذلك تحسن الحالة الصحية العامة لهذه النعاج و حملانها.

مفاتيح الكلمات: البروبوليس و الميثان و الهرمونات و بيولوجيا الدم و و انتاج اللبن و الأستفادة .

SUMMARY

1 INTRODUÇÃO

Estratégias nutricionais visando maximizar a produção animal têm levado ao uso de aditivos dietéticos em ruminantes, como os ionóforos e probióticos (NAGARAJA, 1995). Ionóforos (monensina, por exemplo), o aditivo alimentar mais comum comercialmente, tem sido utilizados para manipular a fermentação ruminal no sentido de menor perda energética (redução na produção de metano, CH_4) e menor degradação proteica com menor produção de amônia (MCGUFFEY et al., 2001; GUAN et al., 2006), o que aumenta a produção de leite, melhorando também a composição desse leite e melhora a saúde dos animais devido ao menor risco de cetose e mastite (DUFFIELD et al., 2008; HAMILTON; MITLOEHNER, 2008).

No entanto nos últimos anos, o uso desses produtos tem enfrentado menor aceitação da sociedade em muitos países, devido ao risco de resíduos destes na carne e leite e ainda a possibilidade de desenvolvimento de linhagens bacterianas resistentes (MATHEW et al., 2001). Em particular, o uso rotineiro de antibióticos para nutrição de ruminantes tem sido criticado pelas organizações de consumidores tendo sido restringido na União Europeia desde 2006 (OEZTUERK; SAGMANLIGIL, 2009). Consequentemente, muitos estudos têm sido conduzidos no sentido de descobrir aditivos alimentares alternativos, principalmente os considerados como produtos naturais, os quais seriam aceitos pelos consumidores.

Entre estes aditivos destacam-se os ácidos orgânicos (KHAMPA; WANAPAT, 2007), enzimas (CHUNG et al., 2012), levedura (STOCKDALE ; GILL, 2011), óleos essenciais (GIANNENAS, et al., 2011; SALLAM et al., 2011) e taninos condensados (ABDALLA et al., 2012; JUHNKE et al., 2012) e recentemente própolis. No entanto esto potencial da própolis para manipulação da fisiologia digestiva e mais especificamente as funções ruminais e repostas fisiológicas não têm sido completamente estudados (ÍTAVO et al., 2011).

Própolis é uma resina encontrada dentro de colmeias que tem sido amplamente usada em medicina popular em muitas culturas, e recentemente esta tem ganhado popularidade em todo mundo como um importante constituinte de fármacos manipulados, alimentos saudáveis e cosméticos (ZHOU et al., 2008). Abelhas (*Apis mellifera*) coletam a própolis das flores e, portanto, a composição química deste depende das características fitogeográficas do local de coleta. As substâncias encontradas na própolis geralmente fazem parte da composição normal de alimentos e aditivos alimentares e dessa maneira são reconhecidas como substâncias GRAS (Geralmente reconhecido como seguro) (BURDOCK, 1998). Mais de 200

constituintes têm sido identificados em amostras de própolis como os flavonoides, isoflavonoides, ácidos aromáticos, terpenos, e compostos fenólicos, os quais parecem ser os principais componentes responsáveis pelo atividade biológica e farmacológica das amostras de própolis (BANKOVA, 2009).

Em diferentes habitats, as abelhas escolhem diferentes espécies de plantas como fontes de própolis e consequentemente a composição química deste produto é altamente variável. No entanto, as diferentes composições químicas sempre apresentam considerável atividade biológica, especialmente antimicrobiana, antifúngica, antiprotozoário, antioxidante, anticarcinogênica e antiviral (KUMAZAWA et al., 2004; SEIDEL et al., 2008). Nos últimos anos, o fornecimento de própolis para ruminantes como alternativa aos antibióticos dietéticos tem obtido certo sucesso, apresentado efeito na fermentação ruminal pelo aumento da concentração de propionato (10,3%), reduzindo emissão de CH_4 assim como a população de protozoário (BRODISCOU et al., 2000), aumentando a produção de leite, rendimento de proteína e conteúdo de gordura no leite (FREITAS et al., 2009; ANDRIGHETTO et al., 2005), além disso aumentando a resistência contra helmintos (PRINCIPAL et al., 2002) e diminuindo a ocorrência de mastites devido a sua atividade antimicrobinana contra *Candida albicans*, *Escherichia coli*, *Staphylococcus sp* e *Streptococcus sp* (SILVA et al., 2012), e ainda se observando melhora na conversão alimentar de bois Nelores (ZAWADZKI et al., 2011).

A própolis brasileira tem sido amplamente estudada visando elucidar suas propriedades biológicas. Por isso, até o momento, 12 tipos de própolis brasileiras têm sido caracterizadas e classificadas em tipos de 1 a 12 (PARK et al., 2000). Um novo tipo de própolis brasileira, popularmente conhecido como “própolis vermelha”, tem se destacado pela sua atividade antioxidante e antimicrobiana em ensaios *in vitro*, se tornando alvo de estudos mais detalhados (ALENCAR et al., 2007). No entanto, existem poucos estudos em relação ao efeito deste novo tipo de própolis como aditivo nutricional de ruminantes sobre a fermentação ruminal, reprodução, desempenho produtivo e saúde animal.

1.1 Hipóteses

A hipótese deste estudo é de que usando a própolis como aditivo dietético natural para ovinos irá alterar a fermentação ruminal permitindo menor perda energética enquanto melhora a resposta produtiva e situação sanitária em um período crítico para ovelhas Santa Inês (cobertura e lactação).

1.2 Objetivos

1.2.1 Objetivo geral

Estudar a fermentação ruminal assim como as propriedades antimetanogênicas *in vitro* da própolis e avaliar o desempenho produtivo e reprodutivo de ovelhas durante os períodos de reprodução e de lactação

1.2.2 Objetivos específicos

- Estudar o efeito de dois tipos de própolis (Brasileiro e Egípcio) em diferentes níveis na produção de gases *in vitro*, emissão de CH_4, parâmetros de fermentação ruminal, degradabilidade verdadeiramente de matéria orgânica e contagem de protozoários
- Estudar o efeito da própolis vermelha brasileira no desempenho reprodutivo de ovelhas Santa Inês incluindo avaliação de hormônios, metabólitos sanguíneos e da resposta parasitária *in vivo*
- Estudar o efeito da própolis na produção e composição do leite e no desempenho dos cordeiros

1.3 Desenvolvimento

Os resultados deste estudo estão apresentados na forma de capítulos. No primeiro capítulo são apresentadas a introdução e a revisão de literatura sobre a própolis e sua ação na fermentação ruminal, reprodução e produção animal. A seguir, são apresentados três experimentos delineados para estudar o efeito da própolis brasileira e egípcia na fermentação ruminal *in vitro* (capítulo 3) e o efeito da administração oral da própolis vermelha brasileira sobre o desempenho reprodutivo e a saúde de ovelhas Santa Inês (capítulo 4) e o efeito da própolis vermelha brasileira na produção e composição do leite e no desempenho dos cordeiros (capítulo 5). Como conclusão geral, este estudo destaca o potencial da propolis de manipular a fermentação ruminal para reduzir produção de CH_4, assim como melhorar a situação sanitária das ovelhas durante a estação de cobertura, além de aumentar a qualidade do leite, quantidade e desempenho dos cordeiros.

A conclusão do trabalho é apresentada na parte final, onde é apresentado que:

- Os principais componentes da própolis vermelha brasileira (PVB) foram os isoflavonóides enquanto que na própolis marrom egípcia (PME) foram os ácidos graxos. Tais componentes são responsáveis pela atividade biológica das respectivas própolis.

- As própolis reduziram a emissão de metano (CH_4) e melhoraram a digestibilidade verdadeira da matéria orgânica (DVMO).

- As própolis foram capazes de melhorar a fermentação ruminal aumentando a produção de ácidos graxos de cadeia curta (AGCC) individual e total sendo promissores agentes de mitigação de CH_4 em ruminantes.

- Administração oral da PVB não prejudicou o desempenho reprodutivo de ovelhas Santa Inês.

- A própolis vermelha brasileira pode agir como agente anti-estresse.

- As própolis tiveram bom impacto sobre a saúde das ovelhas e podem ser usadas como promissores aditivos alimentares durante períodos críticos como o do “flushing”.

- As própolis foram eficientes em controlar helmintos de ovinos.

- A administração oral da PVB aumentou a produção e melhorou a qualidade do leite ovino; podendo atuar no controle da mastite e melhorando o desempenho dos cordeiros.

2 INTRODUCTION

Nutritional strategies targeting to maximize animal production have prompted the use of dietary ruminant additives such as ionophores and probiotics (NAGARAJA, 1995). Ionophores (i, e monensin) the most common commercial feed additives used successfully to manipulate rumen fermentation towards less energy loss (methane CH_4) and protein degradation as ammonia. (MCGUFFEY et al., 2001; GUAN et al., 2006), improves ruminant health by decreasing the risk of ketosis and mastitis during the transition period and enhanced milk production and composition (DUFFIELD et al., 2008; HAMILTON; MITLOEHNER, 2008).

However, in recent years, this practice of using antibiotics has faced reduced social acceptance in many countries, due to the risk of transferring residues into meat and milk and resistant strains of bacteria (MATHEW et al., 2001). In particular, the routine use of antibiotics for livestock nutrition has been criticized by consumer organizations and has been restricted within the European Union 2006 (OEZTUERK; SAGMANLIGIL, 2009). As a consequence, several studies have been recently conducted in order to discover other feed alternative additives which considered to be natural products that consumers would accept, among these additives organic acids (KHAMPA; WANAPAT, 2007), enzymes (CHUNG et al., 2012), yeast (STOCKDALE; GILL, 2011), essential oils (GIANNENAS et al., 2011; SALLAM et al., 2011) and condensed tannins (ABDALLA et al., 2012; JUHNKE et al., 2012) and recently propolis, however its potential for manipulating digestive physiology and more specifically rumen functions and physiological responses has not widely assessed (ÍTAVO et al., 2011).

Propolis (bee glue), is resinous hive product widely used in folk medicine of many nations, is recently gaining popularity all over the world as important constituent of over-the-counter preparations, health foods and cosmetics (ZHOU et al., 2008). Bees (*Apis mellifera*) Collect propolis from plants buds and thus its chemical composition depends on the phytogeographic characteristics of the site of collection. Substances, which are identified in propolis, generally are typical constituents of food and/or food additives, and are recognized as Generally Recognized as Safe (GRAS) substances (BURDOCK, 1998). More than 200 constituents have been identified in different propolis samples such as flavonoids, isoflavonids, aromatic acids, terpenes, and phenolic constituents appear to be the principal components responsible for the biological and pharmacological activities of propolis samples (BANKOVA, 2009). In different habitats, bees choose different plant species as propolis

sources and consequently the chemical composition of this product is highly variable. However, of different chemical composition, propolis always demonstrates considerable biological activity, especially antimicrobial, antifungal, antiprotozoal, antioxidant, anti-cariogenic, and antiviral (KUMAZAWA et al., 2004; SEIDEL et al., 2008; SILVA et al., 2008). Recently, dietary propolis addition for ruminants as alternative to dietary antibiotics (ÍTAVO et al., 2011) was shown to affect rumen fermentation by increasing propionate concentration (10.3%), reducing CH_4 emission as well as protozoa population (BRODISCOU et al., 2000), improve cow milk production, protein yield and buffalo milk fat content (FREITAS et al., 2009; ANDRIGHETTO et al., 2005), improving ruminates resistance against helminthes (PRINCIPAL et al., 2002) decreasing possibilities of mastitis infection by their antimicrobial activity against *Candida albicans*, *Escherichia coli*, *Staphylococcus sp* and *Streptococcus sp* (SILVA et al., 2012) and enhanced Nellore bulls feed conversion ratio (ZAWADZKI et al., 2011).

Brazilian propolis has been widely studied to elucidate its several biological properties. Thus, to date, 12 types of Brazilian propolis have been characterized and classified into types 1–12 (PARK et al., 2000). A new type of Brazilian propolis has recently become the subject of detailed studies, popularly known as “red propolis” demonstrated antioxidant and antimicrobial activities in preliminary *in vitro* assays (ALENCAR et al., 2007; SILVA et al., 2008) however, there are very limited studies concerning the effect of this new type of propolis as ruminant dietary additives on rumen fermentation, reproduction, production performance and animal health.

2.1 Hypothesis

This study hypothesis that, using propolis as dietary natural alternative additives for sheep will alter rumen fermentation towards less diet energy loss while enhancing the physiological responses and health during the most critical periods (breeding and lactation) of Santa Inês ewes.

2.2 Objectives

2.2.1 General objective

Study the rumen fermentation as well as anti-metanogenic properties *in vitro* of propolis (*Apis mellifera*) and reproductive and productive performance of Santa Inês ewes during breeding and lactation period.

2.2.2 Specific objectives

- Effect of two types (Brazilian and Egyptian) propolis with different levels on gas production, CH_4 emission, rumen fermentation parameters and degradability, protozoa count *in vitro*.
- Effect of Brazilian red propolis on reproduction performance general health of Santa Inês ewes including hormones, blood metabolites and parasitic responses *in vivo.*
- Effect of propolis on milk production, composition and lambs performance *in vivo.*

2.3 Development

The results of this study are presented in the form of chapters. The first and second chapters are introduction and the review of the literature on propolis and its action on rumen fermentation, animal reproduction and production. Following there are presented three experiments designed to study the effect of Brazilian and Egyptian propolis on rumen fermentation *in vitro* (chapter 3) and the effect of oral administration of Brazilain red propolis on Santa Inês ewes reproduction performance and general health (chapter 4) and the effect of Brazilain red propolis on milk yield, composition and lambs performance (chapter 5). As general conclusion these studies highlight the potential of propolis to handle the ruminal fermentation in order to reduce the production of CH_4, as well as to improve the health of ewes during the breeding season, besides increasing milk quality, quantity and performance of lambs.

2.4 Literature Review

2.4.1 What is propolis (Bee Glue)?

Propolis has been employed extensively since ancient times. Egyptians benefited from the anti-putrefactive properties of propolis in order to embalm their deads. Propolis was used as an antiseptic and cicatrizant agent by the Greek and Roman physicians. Incas employed propolis as an anti-pyretic agent, and the London pharmacopoeias of the 17th century listed propolis as an official drug. Its use continues today as a popular remedy and is available in either in pure form or combined with other natural products in cosmetics and as a constituent of health foods. Scientists have been interested in the investigation of its constituents and biological properties in the last decades (BANKOVA, 2005; SFORCIN, 2007).

Propolis is a resinous substance collected by honeybees (*Apis mellifera*) from buds and leaves of trees and plants, mixing with pollen which is used to inhibit microorganism growth in their nests and to repair the hives. It may be ochre, red, brown, light brown or green; some are friable and steady, while the others are gummy and elastic (SALATINO et al., 2005; KRELL, 1996). Etymologically the word comes from the Greek, meaning Word propolis (pro = in defense or for, and polis = city) “in defense of the city (or beehive)”. Geopropolis is equivalent to the propolis of the honey bee; it is produced by hymenopterans (Hymenoptera: Meliponinae) native to Brazil, and contains clay soil materials (BARTH, 2006). Substances, which are identified in propolis, generally are typical constituents of food and/or food additives, and are recognized as Generally Recognized As Safe (GRAS) substances (BURDOCK, 1998). Numerous studies have proven its versatile pharmacological activities: antibacterial, antifungal, antiviral, anti-inflammatory, hepatoprotective, antioxidant and antitumoral. (BANSKOTA et al., 2001; ALENCAR et al., 2007).

2.4.2 Chemical composition of propolis

Propolis chemical composition depends on the phytogeographic characteristics of the site of collection as shown in Table 2.1, since bees choose different plants as source of propolis in different habitats (POPOVA et al., 2010). Propolis is composed in general of 45% resins, 30% waxes and fatty acids, 10% essential oils, 5% pollens and 10% organic compounds and minerals (CHENA et al., 2009). More than 300 chemical compounds have

been described in propolis of diverse origins (CASTALDO; CAPASSO, 2002; PEREIRA et al., 2002; 2003). For example, lignans have been identified in samples of propolis obtained from Santa Cruz (VI Region, Chile) (VALCIC et al., 1999).

Table 2.1 - Geographic distribution of propolis with definitions of type, plant origin and main content

Propolis type	Geographic origin	Plant source	Major constituents
Poplar	Europe, North America, non-tropic regions of Asia, New Zealand	*Populus* spp. of section Aigeiros, most often *P. nigra* L.	Flavones, flavanones, cinnamic acids and their esters
Green (alecrim) Brazilian	Brazil	*Baccharis* spp., predominantly *B. dracunculifolia* DC.	Prenylated *p*-coumaric acids, diterpenic acids
Birch	Russia	*Betula verrucosa* Ehrh.	Flavones and flavonols (not the same as in Poplar type)
Red propolis	Cuba, Brazil, Mexico	*Dalbergia* spp.	Isoflavonoids (isovlavans, pterocarpans)
Mediterranean	Sicily, Greece, Crete, Malta,	Cupressaceae (species unidentified)	Diterpenes (mainly acids of labdane type)
"Clusia"	Cuba, Venezuela	*Clusia* spp.	Polyprenylated benzophenones
"Pacific"	Pacific region (Okinawa, Taiwan, Indonesia)	*Macaranga tanarius*	C-Prenyl-flavanones

Source: (SFORCIN; BANKOVA, 2011)

The presence of flavonoids and isoflavonoids has been similarly detected in commercial samples (ASTUDILLO et al., 2000; ALENCAR et al., 2007). The presence of terpenes and flavonoids was demonstrated in samples of propolis that were typified palynologically. Among these, the presence of acacetine, cinnamic acid, cumarin, galangine, izalpine kaempferide pinocembrine, prenyletin, viscidone, and vanillin, have been reported (MUÑOZ et al., 2001a; 2001b).

Prenyletin has been identified in epicuticular wax extracts obtained from *Haplopappus foliosus* (VOGEL et al., 2005). Additionally, galangine, chrysin, and 3-methylgalangin 7-methylgalangin (ALARCÓN BARTOLOTTI, 1989) were identified in samples obtained in Valdivia. The 5,7-dihydroxyfl avone, 3,5,7- trihydroxyfl avone and 5,7-dihydroxyfl avanone showed a strong effect as free radical catchers (ASTUDILLO et al., 2000). Brazilian propolis has been widely studied to elucidate its several biological properties. Thus, to date, 12 types of Brazilian propolis have been characterized and classified into types 1–12 (PARK et al., 2000).

A new type of Brazilian propolis, popularly known as “red propolis” demonstrated antioxidant and antimicrobial activities in preliminary *in vitro* assays (ALENCAR et al., 2007). Several compounds were identified for the first time in Brazilian propolis samples such as methyl o-orsellinate, methyl abietate, 2,4,6-trimethylphenol, homopterocarpin, medicarpin, 4′,7-dimethoxy-2′-isoflavonol and 7,4′- dihydroxyisoflavone. At least four isoflavones, never before reported in propolis, could be observed; such isoflavones as homopterocarpin, medicarpin and 4′,7-dimethoxy-2′- isoflavonol presented the most abundant compounds by the GC–MS technique (ALENCAR et al., 2007). These isoflavones have been reported as having antimicrobial, antifungal, anticancer and antioxidant activity (RUFER; KULLING, 2006).

Silva et al. (2008) concluded that, from the results of UV-VIS spectroscopy, RP-HPTLC, RP-HPLC and GC/MS *Dalbergia ecastophyllum* resin is the botanical source of Brazilian red propolis and that this can be considered a 13th type of Brazilian propolis, complementing the 12 types proposed by Park et al. (2000). This is the first time the botanical origin of a type of propolis is reported as occurring in a species of the leguminosae family, rich in isoflavonoids.

2.4.3 Effect of propolis on gas production, methane emission and rumen fermentation

Until recently, only a few reports were found in the literature dealing with the effects of propolis on gas production and CH_4 emission. Stradiotti Júnior et al. (2004b) observed that propolis extract, when compared to the control treatment, reduced the final total production and the final gas production for fiber carbohydrates. Moreover, Morsy et al. (2011) concluded that green and alamo Brazilian propolis presented a similar reduction on rumen CH_4 production without negative effect on the rumen gas production and degradability of organic matter *in vitro*. In these sense, propolis could be used as a natural alternatives product for chemical ionophores, however more *in vitro* and *in vivo* studies are needed to study the bioactive components of propolis to understand the mode of action of propolis when comparing to monensin. Moreover the ethanol extract of propolis affects fermentation and methanogenesis of continuous microbial culture in ruminants; it decreases protozoa population and increases propionate levels by 10.3% without affecting other ruminal fatty

acids in dual outflow fermenters supplied with a 50:50 orchard grass hay plus barley diet (BRODISCOU et al., 2000).

Stradiotti Júnior et al. (2004) reported that propolis extract increased the total short chain fatty acids (SCFA) concentration in Holstein steers fed a diet containing 65% forage and 35% concentrate. On the other hand, these latter authors underlined that the molar proportions of ruminal SCFA were not changed by propolis treatment. Furthermore, Lana et al. (2005; 2007) showed that supplementation of propolis (up to 6 g/animal/day) did not affect total and individual SCFA concentrations in dairy goats fed a diet of 67% corn silage and 33% concentrate. The reason for non-beneficial effect of propolis on ruminal SCFA may be due to the lack of its inhibitory effect on rumen ciliates.

Rumen ciliates contribute to the greater part of ruminal methanogenesis via hydrogen supply to the endosymbiotic and episymbiotic methanogens (IRBIS; USHIDA, 2004). Inhibition of protozoa reduces CH_4 release by diverting reducing equivalents from CH_4 to propionate synthesis in the rumen (KREUZER et al., 1986). Such a change is nutritionally beneficial to the ruminal energetic metabolism because propionate is the gluconeogenic SCFA and it is more efficiently utilized by ruminant than other SCFA. In Broudiscou's (2000) study propolis (0.5 g/l) did not significantly change the counts of rumen ciliates in dual outflow fermenters. However, Rispoli et al. (2009) found that propolis extract reduced the numbers of ciliates in the rumen of buffaloes fed a diet consisting of corn silage and concentrate (50:50) but not in cattle fed the same diet.

Ozturk et al. (2010) reported that NH_3-N concentrations decreased (P<0.05) in a dose-dependent manner when ruminal fluid was incubated with increasing levels of propolis, which is consistent with previous reports (OLIVEIRA et al., 2004; 2006; STRADIOTTI JÚNIOR et al., 2004a). The ability of propolis to reduce ammonia production by ruminal microorganisms was confirmed by the initial results observed with the use of propolis *in vitro* and *in vivo* (STRADIOTTI JÚNIOR et al., 2001). On the other hand Lana et al (2007) observed that there was no effect of the increasing levels of ethanolic extract of propolis (0, 1.0, 2.0, 4.0, 8.0, and 12.0 ml/animal/day) and ground crude propolis (0, 0.5, 1.0, 2.0, 4.0, and 6.0 g/animal/day) on the NH_3-N concentration and pH.

There are few data about the effect of propolis on ruminal bacteria. However, the antibacterial effect of propolis on different bacterial strains has been shown by several authors such as Alencar et al. (2007) although little is known about the mechanisms of propolis antibacterial action. Propolis is an alternative to the use of dietary antibiotics (ÍTAVO et al.,

2011). Propolis can inhibit the growth of gram positive bacteria; it might be a useful additive for modifying microbial fermentation in the rumen (ÍTAVO et al., 2011).

According to Mirzoeva et al. (1997), propolis has bacteriostatic activity against gram-positive and some gram-negative bacteria. The action mechanism of propolis is likely related to changes in the bioenergetic status of the bacterial membrane, which inhibits bacterial motility. This is similar to the action of ionophores. In addition Takaisi-Kikuni and Schilder (1994) observed that the antibacterial action against *Streptococcus agalactiae* was complex, involving several mechanisms such as the formation of pseudo-multicellular streptococci; disorganization of the cytoplasm, the cytoplasmatic membrane, and the cell wall; partial bacteriolysis; and inhibition of protein synthesis. However, its potential for manipulating rumen microbial fermentation has not been widely assessed (OZTURK et al., 2010).

2.4.4 Effect of propolis on hematological, biochemical and immunity parameters

Unfortunately there were limited data about the effect of long term effect of propolis on hematological and biochemical parameters in ruminants, and most of the data were belong to humans or several animal species (hens, rats and fish). Jasprica et al. (2007) in an *in vivo* study which has been conducted on 47 healthy women and men in order to investigate whether daily intake of one dose powdered propolis extract (0.65 g of dry propolis extract) during 30 days has any influence on the blood parameters, the results showed that the effect of daily propolis intake seems to be time and gender related. For the selected men there was decrease (P<0.007) in red blood cell count (RBC) and hemoglobin (Hb) while mean corpuscular volume (MCV) and red cell distribution width (RDW) were increased (P<0.005). Levels of cholesterol, low-density lipoproteins LDL, high-density lipoproteins HDL, triglycerides, glucose, uric acid, and iron-binding proteins (ferritin, transferrin) did not show any significant fluctuation during the study. For the women test group none of the red blood cell parameters as well as the parameters described above, exhibited any significant change induced by the propolis supplementation.

Cetin et al. (2010b) evaluated the effect of 4 different levels of propolis supplementation (0.5, 1, 3, and 6 g of propolis/kg of diet) on the hematological and immunological parameters of laying hens and they observed that, the level of 3 g/kg diet of propolis increased (P<0.05) RBC compared with the other treatments while, hemoglobin, hematocrit values, total leucocyte (WBC) and differential leucocytes counts were not influenced by propolis

supplementation. The same results were observed by Cetin et al. (2010a) who demonstrated that administration of propolis alone (100 mg/kg BW/day), does not cause any significant alteration on the hematological parameters (RBC, Hb, PCV, WBC and white blood cells proliferation) and biochemical indices including (glucose, AST, ALT, ALP, Total Cholesterol, Triglycried, Total protein and Bilirubin) in female Wistar–Albino Rats blood.

Fuliang et al. (2005) concluded that, propolis decreased ($P < 0.05$) levels of blood glucose (FBG), fructosamine (FRU), malonaldehyde (MDA), nitric oxide (NO), nitric oxide synthetase (NOS), total cholesterol (TC), triglyceride (TG), (LDL), very low-density lipoprotein (VLDL) in serum of fasting rats; and increased ($P < 0.05$) serum levels of HDL and superoxide dismutase (SOD). This suggests that propolis can control blood glucose and modulate the metabolism of glucose and blood lipid, leading to decreased outputs of lipid peroxidation and scavenge the free radicals in rats with diabetes mellitus.

Talas and Mehmet (2009) observed an increasing ($P < 0.05$) on WBC, MCV, MCH and granulocytes values on rainbow trout fish (*Oncorhynchus mykiss*) treated with 0.02 and 0.03g/L propolis while a decrease ($P < 0.05$) in RBC, Hb and PCV for fish exposed to 0.02and 0.03g/L propolis also in the same experiment of propolis caused an increase ($P< 0.05$) in the concentrations of glucose, blood urea nitrogen, triglyceride, total cholesterol, lactated hydrogenase, amylase and gamma glutamyl transferase. But, there was a significant decrease ($P < 0.05$) in the concentrations of aspartate aminotransferase and alkaline phosphatase.

Orsolić and Basic (2003; 2005) reported that water-soluble derivatives of propolis (WSDP) given to mice caused a significant elevation of leucocytes in peripheral blood and increased ($P < 0.05$) proliferation of WBC precursors from pluripotent stem cell in mice. Moreover, Eraslan et al. (2007) found a significant increase-compared to control group (zero propolis) in the total protein values of the rats that fed propolis at 200 mg/kg BW and drinking water for 21 days. In addition Fuliang et al. (2005) reported that propolis caused a decrease in triglyceride levels when administered to rats with diabetes mellitus, the same results were observed by Kolankaya et al. (2002) for rats treated with alcohol.

While and Kashkooli et al. (2011) concluded that propolis have no significant alterations in the serum levels of total protein, albumin, globulin, LDL, HDL, triglycerides and activities of glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, alkaline phosphatase and lactate dehydrogenase, when compared to the control (zero propolis) group.

Several studies on the immunomodulatory activities of water-soluble derivatives of propolis (**WSDP**) have been published (TAKAGI et al., 2005; FISCHER et al., 2007), but

little is known about the effect of ethanolic extracts of propolis on the immunological variables in ruminants. Yaghoubi et al. (2008) found that, serum immunoglobulin G (IgG) concentrations remained lower at the first 3 weeks of the experiment when calves received low dose of propolis flavonoids extract (7.3 10^{-5} g/kg BW) but not at the high dose (3.6 10^{-3} g/kg BW). At week 4, both medium (7.3 10^{-4} g/kg BW) and low doses of propolis extract moderated serum IgG. At week 8, the medium and high dose of propolis extract lowered serum IgG. At week 6, calves fed high and medium propolis extract doses had lower blood immunoglobulin M (IgM) than control calves. These results suggest that propolis extract affect the humoral immune response and can improve growth in young calves and this response depended on calf age.

Cetin et al. (2010b) observed that, the addition of propolis at 3 g/kg diet resulted in increases ($P < 0.05$) in the serum IgG and IgM levels and significant decreases ($P < 0.05$) in the peripheral blood T-lymphocyte percentage compared with those of the control (diet without propolis addition). With regards to the humoral immune response, the ethanolic extract of propolis (500µg/mouse) increased the antibody production in sheep red blood cells (RBC) immunized mice (SCHELLER et al., 1998).

Several studies have showed that propolis enhance the immune system in different animal species by increasing macrophage activity (PARK et al., 2004), interleukin-1, interleukin-2, and interleukin-4 levels (ORSOLIĆ; BASIC, 2003; PARK et al., 2004). These cytokines stimulate B lymphocytes and then they are changed to plasma cells, which would be able to produce immunoglobulins (DIKER, 1998). *In vitro* and *in vivo* assays demonstrated the modulatory action of propolis on murine peritoneal macrophages, increasing their microbicidal activity. Its stimulant action on the lytic activity of natural killer cells against tumor cells, and on antibody production was demonstrated.

Propolis inhibitory effects on lymph proliferation may be associated to its anti-inflammatory property. In immunological assays, the best results were observed when propolis was administered over a short-term to animals (SFORCIN, 2007).

2.4.5 Effect of propolis on parasites

Propolis demonstrated promising efficacy to control sheep helminthiasis and decrease ($P < 0.05$) fecal egg count (FEC) in treated group compared with control (PRINCIPAL et al., 2002). Moreover, Heinzen et al. (2012) concluded that the anthelmintic effect of alcoholic

extract of propolis (30%) in naturally infected calves where 83% of animals showed a decrease of 48.48% of FEC specially (*Trichostrongylus* sp. and *Strongyloides* sp). Principal et al. (2002) tested several concentrations of propolis extracts to control of helminthiasis in West African sheep and found that dose of 10 mL of 30% ethanolic extracts of propolis was the most effective in this species.

Araújo et al. (2006) and Castagnara et al. (2007) found a reduction of FEC in Santa Inês sheep breed when treated with 30% extract of propolis. Loureiro (2007) found the addition of 30 mg/kg diet of propolis extract (30%) to the diet was effective in reducing the number of FEC of sheep, demonstrating its effect antiparasitic and the possibility of its use in control of worms. Dürrewald et al. (2008) used alcoholic extract of propolis at 33% for 20 cattle (twelve months age) naturally infected with *Trichuris* sp., *Tricostrongilos* sp. and *Ascaris* sp. administered in single dose once or twice a day for three days consecutive, and 30 days after the start of treatment, resulted in a reduction of 59.7% in the FEC of animals receiving propolis treatment while the FEC was increased by 63.6% in the control animals that did not receive propolis.

Propolis presented various biological and therapeutic activities, which had been associated with the presence of flavonoids, isoflavonoids, aromatic acids and esters (SALOMÃO et al., 2004). Among the several biological activates of propolis, it possesses antiparasite activities (HIGASHI; DE CASTRO, 1994; SALOMÃO et al., 2004; FREITAS et al., 2006). It had been proven to be 100% effective against some lethal protozoa and would also decrease inflammation associated with parasite infection (HIGASHI; DE CASTRO, 1994). The Egyptian propolis (collected from Siwa) showed highly inhibitory activity as well as triclabendazole (TCBZ) (‘Fasinex’,Ciba-Geigy) on the vitality and hatchability of immature *F. gigantica* eggs when three different concentrations of propolis (10, 20 and 30 mg/mL) incubated with adult *F. gigantica in vitro* for 24 h. These results suggested that propolis could be an effective alternative anindicate of the fasciolicidal drugs (HEGAZI et al., 2006; 2007).

2.4.6 Effect of propolis on reproduction

Flavonoids are major functional components of many herbal and insect preparations for medical use, e.g., propolis (bee’s glue) and honey (HAVSTEEN, 2002). It was ascribed to the fact that the steric positions of the hydroxyl group in this flavonoid are almost identical to

those of estrogens. Flavonoids, due to their nonpolarity or in complex with serum albumin, can pass the plasma membrane and can attach to the cytoplasmic steroid receptor. Accordingly, flavonoids are carried into the cell nucleus to the transcription complex at the genes controlling the expression of estrogen receptors (ERs) and perhaps also of other proteins participating in the growth reproduction and function of the mammary gland.

Flavonoides can act as hormones in both plants and animals (BAKER, 1998). The estrogenic effect of some flavonoids originally was discovered by observing the behavior of sheep that had eaten fermented clover became sexually aroused (SONNENBICHLER et al., 1980). Since estrogens also have anabolic effects (SOKOLOVA et al., 1978), one might suspect that flavonoids might be able to act as growth hormones in animals also. However, so far only few indications of such a function have been found (HAVSTEEN, 2002).

Flavonoids and isoflavonoids able to display an estrogenic and pregnancy inhibitory function (JAIN et al., 1993). This effect was already known by lay practitioners in the middle ages, but it now has been confirmed using modern methods. After ovariectomy, endothelial dysfunction resulting from the lack of estrogen can be improved by the supplementation with either genistein or 17-β-estradiol (SQUADRITO et al., 2000). The authors concluded that the effects of the two substances were overlapping. However, the discussion of the phyto-estrogenic effect of flavonoids in the literature has been rather controversial, especially regarding possible replacement therapy with genistein and estrogen to improve endothelial dysfunction (SQUADRITO et al., 2000). On the other hand some flavonoids also inhibit the hyaluronidase in sperm, which facilitates the entry of the spermatocyte into the oocyte (LI et al., 1997).

2.4.7 Effect of propolis on milk yield and composition

Limited studies been done in order to investigate the effect of propolis ethanolic extract in the dairy ruminants' diets on milk yield and composition. Holstein cows fed propolis ethanolic extract in the diet at 30% w/v produced higher significant ($P < 0.05$) milk production, production of milk corrected to 3.5%, protein and fat content and protein production in milk when compared with cows fed the same diet without propolis supplemnation but no significant differences were observed on lactose%, fat% and total solids (STELZER et al., 2009).

Supplementation of lactating West African goats diet in the tropical environment with different levels of Bee Wax Residue Meal which content propolis resulted in significant ($P < 0.05$) increase of milk yield also increased butter fat, protein, calcium and phosphorus contents. The potassium sodium and iron contents were significantly ($P < 0.05$) highest in trated animals compared with other goats fed the same diet without propolis addition (ADEWALE et al., 2010). On the other hand Stelzer et al. (2009) used two levels of the concentrate (20 and 40% dry matter) and presence or absence of propolis ethanolic extract in the diet at 30% w/v for Holstein dairy cows did not alter the intake, digestibility and performance of cows producing above 20 kg of milk/day. Same finding was observed by Lana et al. (2005) who demonstrated that, Alpine dairy goats which supplemented with 10 mL of ethanolic extract of propolis/animal/day, in the diets resulted no significant differences in milk yield, milk yield corrected for 4% fat, fat %, protein %, lactose %, total solids.

The antimicrobial activity of propolis ethanolic extract against *Candida albicans*, *Escherichia coli*, *Staphylococcus sp* and *Streptococcus sp,* the major microorganisms (somatic cell counts) that causing mastitis was observed *in vitro* as well as *in vivo* even in ovine or bovine species (MERESTA et al., 1989; PINTO et al., 2001; LOGUERCIO et al., 2006; SILVA et al., 2012). On the other hand Vargas et al. (2002) and Freitas et al. (2009) concluded that propolis did not affect on somatic cell counts.

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3 RUMEN FERMENTATION AND METHANE PRODUCTION ARE INFLUENCED BY THE *IN VITRO* ADDITION OF BRAZILIAN AND EGYPTIAN PROPOLIS

Abstract

This study was carried out to investigate the chemical composition and effects of Brazilian red propolis (BRP) and Egyptian brown propolis (EBP) on rumen fermentation and methane (CH_4) production using an *in vitro* rumen fermentation method based on semi-automatic gas production system. Ruminal inoculum collected from six rumen cannulated Santa Inês wethers. The substrate was a diet containing 50% Tifton (*Cynodon* spp) hay and 50% concentrate mixture. Monensin (MON) was incubated as a positive control at 3 mM concentration. Propolis extracts were prepared using 70% ethanol and four levels (0, 25, 50 and 100 μg / 75 ml of buffered rumen fluid) of each propolis were tested. The chemical profiles of the propolis by GC/MS showed that the major compounds of the BRP were isoflavonoides especially vestitol (24.62%), propanoic acid (16.49%) and medicarpin (13.52%), while fatty acids especially palmitic acids (24.42%), 3-(Hydroxymethyl)-1-Phenyl-1-Heptadecyn-3-Ol (13.75%) and flavone (10.15 %) were found in EBP. Monensin supplementation reduced ($P<0.001$) gas and CH_4 production with adverse effect on truly degraded organic matter (TDOM) and increased partitioning factor (PF) compared with the control diet (CTL). Both $BRP_{50\mu g}$ and $EBP_{25\mu g}$ showed similar significant effects on CH_4 as MON; and the lowest ($P<0.001$) CH_4 production (14.2 ml/TDOM g) was observed at these concentrations compared with control diet (CTL: 19.3 ml/TDOM). There were no significant ($P>0.05$) differences among treatments (BRP, EBP and MON) when compared with CTL for pH, NH_3-N and TDDM, while both types of peopolis reduced ($P<0.002$) protozoa counts. Monensin supplementation increased ($P<0.001$) the propionate concentration and decreased the acetate/propionate ratio, but BRP and EBP increased ($P<0.001$) acetate, propionate and butyrate concentrations when compared to the control. These results suggest that propolis may be used as natural alternative for chemical ionophores not only to reduce CH_4 but also to enhance the rumen fermentation and OM degradability *in vitro*.

Key words: Gas production. Propolis. Monensin. Fermentation parameters.

3.1 Introduction

The symbiotic relationship between the ruminant animal and the rumen microorganisms has energy loss (methane) and protein inefficiencies (loss as ammonia N) (VAN NEVEL; DEMEYER, 1988). The use of antibiotics as feed additives, such as ionophores had proved to be a useful tool to improve the diet energy and protein utilization (TEDESCHI et al., 2003). However, in recent years, this practice has faced reduced social acceptance due to the risk of transferring residues into meat, milk and resistant strains of bacteria (OEZTUERK; SAGMANLIGIL, 2009). Thus, there is an increasing interest in exploiting natural products that consumers would accept as manipulators of ruminal fermentation such as propolis.

Propolis is a resinous substance collected by honeybees (*Apis mellifera*) from buds and leaves of trees and plants, mixing with pollen as well (ZHOU et al*.,* 2008). Propolis chemical composition depends on the phytogeographic characteristics of the site of collection. In different habitats, bees choose different plant species as propolis sources and consequently the chemical composition of propolis is highly variable. Although propolis presented different chemical composition, numerous studies have proven its versatile pharmacological activities such as: antibacterial, antifungal, antiviral, antiprotozoal, anti inflammatory, hepatoprotective, antioxidant and antitumoral (SEIDEL et al., 2008).

The higher antimicrobial activity of propolis against gram positive than gram negative bacteria (GONSALES et al., 2006) lead to improve the feed efficiency because gram positive bacteria produce more ammonia, hydrogen, and lactate than gram negative species (RUSSELL; STROBEL, 1988), also the antioxidant activity of propolis components (ALENCAR et al., 2007) would lessen oxidative stress, thus promoting better conditions for rumen microbial growth consequently enhancing the fermentation process (CATTANI et al., 2012). In these sense, propolis diet supplementations might be a natural useful alternative to antibiotics supplementation for modifying microbial fermentation in the rumen to reduce the loss of energy as methane, and nitrogen as ammonia from the diet. However, its potential for manipulating rumen microbial fermentation has not been widely assessed. Therefore, the objective of this study was to evaluate the chemical and fermentative effects of two types of propolis collected from two different sites (Brazilian red Propolis and Egyptian brown propolis) on methane production and rumen microbial fermentation characterstics in a short-term *in vitro* study.

3.2 Materials and Methods

3.2.1 Origin of propolis

Brazilian red propolis (BRP) samples were collected in January 2011 from the mangrove region in Marechal Deodoro, a city in the vicinity of Maceio, capital of Alagoas State, in Northeastern Brazil, wet tropical climate (**9° 42′ 36″ S, 35° 53′ 42″ W**) and classified as type 13 according to Park et al. (1998). Egyptian brown propolis (EBP) samples were collected in March 2011 from experimental apiary at Alexandria Governorate, Northwestern Egypt, dry subtropical climate (**31° 11′ 53″ N, 29° 55′ 9″ E**) . The collected samples were weighted and stored separately in the refrigerator at 4 °C until analysis and evaluation.

3.2.2 Preparation of propolis extract

Propolis extraction was done according to Alencar et al. (2007) with some modification. Crude propolis samples (10g) for each type were treated with liquid nitrogen and then grounded to a fine powder, which mixed with 100 mL ethanol 70%. The mixture was transferred to ultrasonic equipment (Kerry, ultrasonic limited model, PUL, 250, England) for 30 minutes. The ethanol extract solution was subsequently filtered through a filter paper (Whatman # 41). The extracted solution was incubated in freezer (-5°C) over night and filtered again. The supernatant was transferred to the rotary-evaporator at approximately 42°C for 30 min to remove all the ethanol. The concentrated extract recovered in the volumetric flask was lyophilized for 3 days to get the resultant brownish resin (pure propolis).

3.2.2.1 Propolis analysis and chemical composition

All chemical analysis of propolis was done at Department of Agri-Food Industry, Food and Nutrition, College of Agriculture “Luiz de Queiroz”, University of São Paulo (USP), Piracicaba, SP, Brazil.

The BRP and EBP samples were chemically analyzed after methylation of the extracts. About 5 mg of each propolis extract was mixed with 75 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Sigma-Aldrich) in a sealed glass tube for 15 min at 60°C to form TMS derivatives as described by Markham et al. (1996). Samples of the methylated solutions were analyzed by Gas Chromatography–Mass Spectrometry (GC-MS) according to Fernández et al. (2008) with some modifications. A Shimadzu Model GC 2010 Series gas chromatograph coupled with shimadzu series mass-selective detector quadrupole mass spectrometer model GCMS-QP 2010 plus was used. Samples were separated on a 30 m × 0.25 mm i.d., 0.25 μm film thickness, capillary column (RTX5MS). The column temperature was initially held at 80 °C for 1 min, and then the temperature was raised to 320 °C at a rate of 5 °C min-1, followed by isothermal period of 20 min. Ultra-high-purity helium carrier gas at a flow rate of 1.0 mL/min. The injector was heated to 280 °C and was on split mode with a split ratio of 1:20, and the injection volume was 1.0 μL. The MSD was acquiring data in the full scan mode (mass range 40-800), with a multiplier voltage of 1000 V and ionization energy of 70 eV. The GC–MS peaks were identified by comparison with data from literature Piccinelli et al. (2005) and the profiles from the Nist 98 library.

3.2.3 *In vito* gas production assay

The chemical analysis and *in vitro* assays were done at the Laboratory of Animal Nutrition, Centre for Nuclear Energy in Agriculture (CENA), University of São Paulo (USP), Piracicaba, Brazil and all treatments and techniques used were in accordance to the Internal Commission for Environmental Ethics in Experimentation with Animals of the Centre of Nuclear Energy in Agriculture (CENA / USP)

3.2.4 Experimental description and treatments

Random regression design was used with inoculum considered as repeated 3 times. In each inoculm (2 rumen cannulated animals), twelve bottles were used per treatment and the mean of the twelve bottles was considered the replicate. Treatments were defined as, blank:

bottles without substrate and containing inoculum plus medium; negative control (CTL): bottle containing substrate + inoculum + medium + ethanol; positive control of monensin (MON): control + 0.156 mg of MON. Four levels of each propolis (BRP and EBP) 0, 25, 50 and 100 μg ethanol extract of propolis / g DM substrate + 25 mL inoculum + 50 mL medium + ethanol extract of propolis.

A stock solution of monensin (M5273; Sigma Aldrich Co., St. Louis, MO; MW = 692.85) was prepared by diluting 15.6 mg in 1.0 mL of pure ethanol which was stored at −10°C until used. Stock solution (10 μL) were added to each bottle (50 mL of medium plus 25 mL of inoculum) to achieve a final monensin concentration of 2.08 mg/L of buffered rumen fluid. According to Selje-Assmann et al. (2008), 11.25 μl of ethanol in 75 mL of buffered rumen fluid had no effects on fermentation (ARAUJO et al., 2011) but ethanol was included in the other treatments.

The substrate was total mixed ration, which composed from 50:50 (w/w) concentrate: forage diet consisting of 50% of Tifton hay *(Cynodon. spp*), 32.7% ground corn, 15.0% soybean meal, 1.0% limestone, and 1.3% mineral premix of on dry matter (DM) basis and was to pass a 1 mm screen.

The substrate sample was chemically analyzed on dry matter (g/kg DM) basis according to AOAC (2006) as for organic matter (OM=923); crude protein (CP as 6.25×N=151) and ether extract (EE=18). The neutral detergent fiber (aNDFom=548), acid detergent fiber (ADFom=256) and acid detergent lignin (ADL= 25.6) according to Van Soest et al. (1991) and adapted to Mertens (2002). The ADFom and aNDFom were assayed with a heat stable amylase and expressed exclusive of residual ash. The ADF was obtained by sequential extraction of NDF residue and ADL was determined by solubilization of cellulose with sulphuric acid exclusive residual ash.

3.2.4.1 Inocula donors and preparations

Six adult rumen cannulated Santa Inês sheep (60 ± 2.5 kg of BW) grazing tropical grass pasture signal grass (*Brachiaria decumbens*), elephant grass (*Pennisetum purpureum*) and supplemented with 180 g/d ground corn 78 g/d of soybean meal were used as donors of rumen content. Animals had free access to a mineral premix and fresh water. Three inocula were used (two animals/each inoculum) at the same incubation time.

Rumen liquid and solid fractions were collected separately from each animal before morning feeding and kept in pre-warmed thermo containers under anaerobic conditions. A liquid fraction was obtained by inserting a tube attached to a 60 mL syringe. Similar volumes (500:500 v/v) of both fractions for each two animals were blended for 10 s, squeezed through four layers of cheesecloth, and maintained in a water bath (39 ^{o}C) under CO_2 flushing. Final three inocula consisted of a mixture of the two samples collected from each two animals.

3.2.4.2 Gas production conditions

An *in vitro* gas production technique (THEODOROU et al., 1994) was adapted to a semi-automatic system (BUENO et al., 2005) using a pressure transducer and data logger (Pressure Press Data 800, LANA, CENA/USP, Piracicaba, SP, Brazil). Serum glass bottles of total volume 160 mL and head space 85 mL were filled sequentially with 500 mg of air dried substrate, 50 mL of incubation medium (Theodorou's medium described in PRESTON, 1995) and 25 mL of inoculum. Bottles were sealed immediately with 20 mm butyl septum stoppers (Bellco Glass Inc, Vineland, NJ, USA), manually mixed, and incubated in a forced air oven (Marconi MA35, Piracicaba, SP, Brazil) at 39^{o}C for 24 h.

Head space gas pressure was measured at 4, 8, 12 and 24 h of incubation. For CH_4 determination, 2.5 mL gas were sampled at each time using a 5 mL syringe (Becton Dickson Indústria Cirúrgica LTDA, Curitiba, PR, Brazil) and stored in a 10 mL vacuum tube. After each gas sampling, bottles were vented, mixed and returned to the oven.

3.2.4.3 Analysis and calculations

3.2.4.3.1Gas and methane emission

Gas production was calculated by the equation defined for our laboratory conditions as:

$$V = 6.6193 - 0.0236 \times p \qquad (n=500;\ r^2 = 0.98)$$

Where: V = gas volume (mL); p = measured pressure (psi). Total gas production in 24 h of incubation was considered as the sum of partial gas production at each time interval. Net gas, expressed as mL/g OM degraded, was calculated by correcting the values of gas production and OM degradation for the corresponding blank. Methane concentration was determined using a gas chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD, 1.0 mm ID, 1 m length; Ref. n^{o} 19809; Restek, Bellefonte, PA, USA). Temperatures of column, injector, and flame ionization detector were 60, 200, and 240ºC, respectively. Helium at 10 mL/min was used as the carrier gas. Methane concentration was determined by external calibration using an analytical curve (0, 30, 60, 90 and 120; mL/L) prepared with pure CH_4 (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brazil; 995 mL/L purity). Methane production was calculated according to Tavendale et al. (2005) as follows:

CH_4, mL = (Total gas, mL + Headspace, 85 mL) × CH_4 concentration, mL/mL. Net CH_4 production, expressed as mL/g OM degraded, was calculated by correcting values of CH_4 production and OM degradation for the corresponding blank.

3.2.4.3.2 Truly degraded organic matter (TDOM) and rumen fermentation characteristics

After 24 h, all the bottles were placed in cold water (4^{o}C) in order to stop the fermentation process. For the TDOM determination, half of those bottles were followed by immediate addition of neutral detergent solution (70 mL) (VAN SOEST et al., 1991) without heat stable α-amylase and incubated at 105^{o}C for 3 h The residue was filtered in pre-weighed crucibles, washed with hot water and acetone and oven dried at 105^{o}C for 16 h. TDOM was determined after ashing at 550^{o}C for 4 h with correction for the corresponding blank.

The NH_3-N concentration measured according to Preston (1995). Protozoa were counted microscopically (Olympus biological microscope, model CX31RBSFA, Philippines) following the procedure described by Dehority et al. (1983). The short chain fatty acids were determined by gas-liquid chromatography (GC HP 5890 Series II/ integrator HP 3396 Series II/automatic injector HP 6890 Series, Agilent Technologies, Palo Alto, CA, USA) according to Palmiquist and Conrad (1971). The internal standard was 2-metylbutyric acid. Each tube contained 100 μL of internal standard, 800 μL of sample, and 200 μL of formic acid. A mixture of SCFA with known concentrations was used as external

standard for the integrator calibration. The partitioning factor (PF) was calculated as the ratio between mg of organic matter truly degraded and gas volume (mL) at 24 h of incubation according to BLUMMEL et al. (1997).

3.2.5 Statistical analysis

Data were subjected to analysis of variance (ANOVA), using the General Linear Model procedure of the SAS software package (2002). The used model was: $Y= \mu+ Fi + e$, where μ is overall mean, Fi the treatment effect and e errors. The significant differences between individual means were identified using Duncan test. Differences were considered significant if $P < 0.05$.

3.3 Results

3.3.1 Propolis major constituents

The chromatograms of GC—MS of Brazilian red propolis (BRP) type 13 and Egyptian brown propolis (EBP) are shown in Figure 3.1. and Table 3.1. The relative percentage of the identified major compounds of the Brazilian propolis were isoflavonoides especially vestitol (24.62%), propanoic acid (Bis[(Trimethylsilyl)Oxy], trimethylsilyl ester) (16.49%) and Medicarpin (13.52%) . The fatty acids especially hexadecanoic acid or palmitic acids (24.42%), 3-(Hydroxymethyl)-1-Phenyl-1-Heptadecyn-3-Ol (13.75%) and flavone (10.15%) were detected in Egyptian propolis.

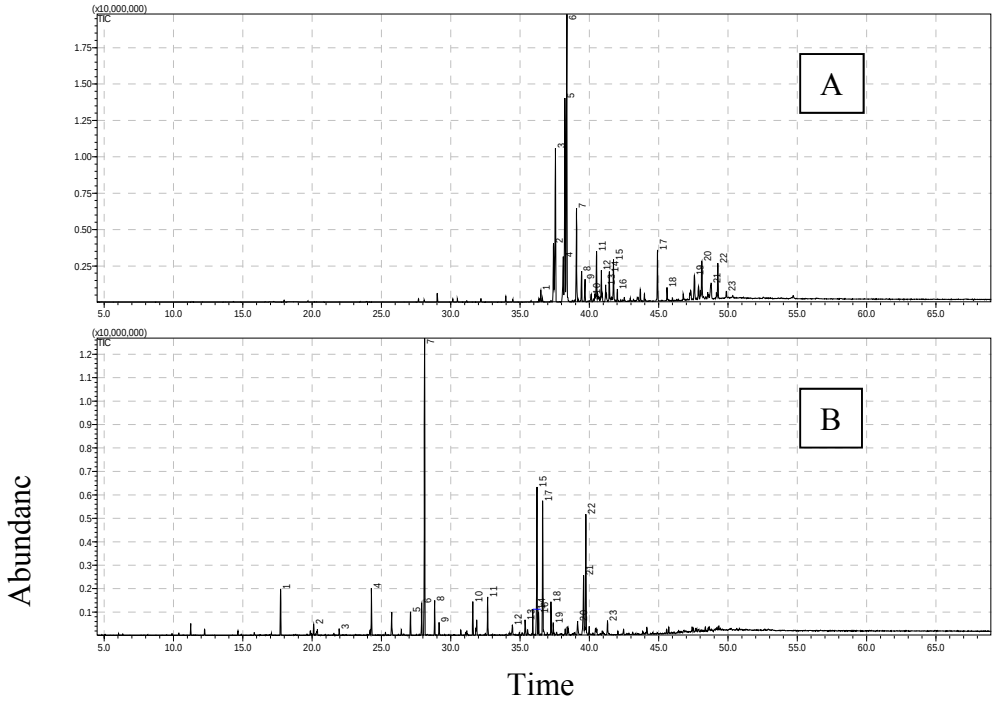

Figure 3.1 - GC-MS chromatograms of two types of propolis (A) Brazilian red propolis and (B) Egyptian brown propolis

Table 3.1 - Identification of constituents from two types of propolis Brazilian red propolis and Egyptian brown propolis by gas chromatography/mass spectrometry

	Brazilian red propolis			Egyptian brown propolis		
Peaks	Compounds	*t*R (min)*a*	(% area of each component)*e*	Compounds	*t*R (min)*a*	(% area of each component)*e*
1	4,4' Bis[(Trimethylsilyl)Ethynyl]-2,2'-Bithiophene-5,5' Dicarbaldehyde	36.49	0.80	Trimethylsilyl 3-Phenyl-2-Propenoate	17.72	3.71
2	Silane, Trimethyl[5-Methyl-2-(1-Methylethyl)Phenoxy]*	37.41	4.97	Dodecanoic Acid, Trimethylsilyl Ester	20.11	0.69
3	Medicarpin	37.55	13.52	6,7-Dihydroxycoumarin Di-Tms	21.95	0.51
4	Benzenepropanoic Acid, 3,4-Bis[(Trimethylsilyl)Oxy]-, Trimethylsilyl Ester*	38.11	3.93	Tetradecanoic Acid, Trimethylsilyl Ester	24.28	3.58
5	Benzenepropanoic Acid, 3,5-Bis[(Trimethylsilyl)Oxy]-, Trimethylsilyl Ester	38.24	16.49	Hexadecanoic Acid, Ethyl Ester	27.09	1.78
6	Vestitol	38.37	24.62	Cinnamic Acid, 3,4-Dimethoxy-, Trimethylsilyl Ester	27.90	2.56
7	4,4'-Bis[(Trimethylsilyl)Ethynyl]-2,2'-Bithiophene-5,5'-Dicarbaldehyde	39,07	7.07	Hexadecanoic Acid, Trimethylsilyl Ester	28.11	24.42
8	Hydrocinnamic Acid, P-(Trimethylsiloxy)-, Trimethylsilyl Ester*	39.43	1.96	Ferulic Acid	28.84	2.68
9	3,4-Dihydroxy-9-Methoxypterocarpan	39.68	1.51	Silane, [1-(5-Ethenyltetrahydro-5-Methyl-2-Furanyl)-1-Methylethoxy]Trimethyl-, Cis*	29.16	1.06
10	3,8-Dihydroxy-9-Methoxypterocarpan (3-Hydroxy-8,9-Dimethoxypterocarpan)	40.13	0.79	Octadecanoic Acid, Trimethylsilyl Ester	31.59	2.60
11	9h-Fluorene-4,5-Diamine, N,N,N',N'-Tetramethyl*	40.51	2.88	3-Methyl-3-Butenyl Isoferulate-Tms-Derivative*	32.66	2.96
12	Formononetin	40.86	1.84	2-Propen-1-One, 1-(2,6-Dihydroxy-4-Methoxyphenyl)-3-Phenyl-, (E)*	34.44	0.84
13	Silane, 9h-Fluoren-9-Ylidenebis(Trimethyl)*	41.17	0.78	1,2,4-Tris(Tert Butyldimethylsiloxy)Naphthalene	35.36	1.57
14	2,5,?-Tri-Oh-Phenylacetate 4tms	41.44	1.77	1h-Imidazole-4-Carboxamide, 5-Amino-, Tetrakis(Trimethylsilyl) Deriv.	35.91	1.94
15	Isoliquiritigenin	41.74	2.64	3-(Hydroxymethyl)-1-Phenyl-1-Heptadecyn-3-Ol	36.22	13.75
16	2-Propenoic Acid, 3-(3,4,5-Trimethoxyphenyl)-, Methyl Ester*	42.02	0.70	2',4',6'-Tris(Trimethylsilyloxy)Chalcone	36.33	1.73
17	Silane, Trimethyl[1-[(Trimethylsilyl)Ethynyl]-2-Naphthalenyl]*	44.91	3.30	4-Quinolinamine, N,3-Diphenyl-2-(Phenylmethyl)	36.63	11.03
18	Propanedioic Acid, Bis[(Trimethylsilyl)Oxy]-, Bis(Trimethylsilyl) Ester*	45.60	0.78	Bis-O-Trimethylsilyl-Palmitinic Acid-Glycerin-(1)-Monoester	37.23	2.65
19	Beta-Amirin Trimethylsilyl Ether	47.57	2.33	Benzoic Acid, 2-[(Trimethylsilyl)Amino]-3-[(Trimethylsilyl)Oxy]-, Methyl Ester*	37.39	0.96
20	Silane, (9,19-Cyclo-9.Beta.-Lanost-24-En-3.Beta.-Yloxy)Trimethyl*	48.11	3.09	1h-Pyrrole, 5-(3-Methoxyphenyl)-2,3-Diphenyl*	39.15	1.61
21	9,19-Cyclolanostan-3-Ol, 24-Methylene-, (3.Beta.)*	48.78	1.21	2-Methyl-1,6-Bis[(Trimethylsilyl)Oxy]Anthra-9,10-Quinone	39.58	5.95
22	Lup-20(29)-En-3-Yl Acetate	49.26	2.54	3,5,7-Tris(Trimethylsilyloxy)Flavone	39.74	10.15
23	Solanesol	49.88	0.48	Meso-Dimethyl-R-3,C-6-Bis[(Tert-Butyldimethylsilyl)Oxy]-T-4,5-Epoxycyclohex-1-Ene-1,2-Dicarboxylate*	41.30	1.27

*t*R (min)*a* = retention time, min) * Components were similarity more than 50% of the search results by comparison with data from literature and the profiles from the Nist 98 library.

3.3.2 Methane and gas production

Table 3.2 data showed that monensin had decreased (P<0.001) net gas production either expressed as mL/g DM or mL/g DOM compared with both CTL and all propolis treatments. Also $BRP_{50\mu g}$ treatment showed significant (P<0.001) decline in gas production either expressed as mL/g DM or mL/g DOM compared with CTL, but there were no differences observed among the propolis levels.Regarding to methane emission, MON showed higher (P<0.001) decrease of net CH4 production (mL/ g DM) compared to the CTL and all BRP treatments, but there were no significant differences observed when compared to all EBP treatments. Monensin inhibited (P<0.001) methane production (mL/ g TDOM) by 44.5% in comparison with CTL and $BRP_{25\mu g}$, while $BRP_{50\mu g}$ and $EBP_{25\mu g}$ inhibited (P<0.001) CH_4 production by 26.5% compared with CTL.

Table 3.2 - Effect of inclusion Brazilian Red Propolis (BRP) and Egyptian Brown propolis (EBP) on gas and methane production *in vitro*

	GP		CH_4		
Additives	**mL/gDM**	**mL/gDOM**	**mL/gDM**	**mL/gTDOM**	**inhibition %**
CTL	142.9^{a}	208.3^{a}	14.4^{a}	19.3^{a}	-
Mon	105.3^{c}	151.8^{c}	7.9^{b}	11.3^{c}	44.5
$BRP_{25\ \mu g}$	131.7^{ab}	184.7^{ab}	11.9^{a}	16.8^{ab}	12.9
$BRP_{50\ \mu g}$	127.8^{b}	177.5^{b}	13.2^{a}	14.2^{bc}	26.4
$BRP_{100\ \mu g}$	131.2^{ab}	184.4^{ab}	11.6^{a}	16.0^{abc}	17.0
$EBP_{25\ \mu g}$	132.2^{ab}	182.0^{ab}	10.3^{ab}	14.2^{bc}	26.4
$EBP_{50\ \mu g}$	134.2^{ab}	191.4^{ab}	10.6^{ab}	14.9^{abc}	22.7
$EBP_{100\ \mu g}$	132.7^{ab}	189.9^{ab}	10.9^{ab}	15.6^{abc}	19.1
SEM	13.303	28.711	3.301	4.905	-
*P.*value	<0.001	<0.001	<0.001	<0.001	-

SEM =stander error of the mean.

^{a,b,c} means with different superscripts, within column, are different (Duncan test, P<0.05) CTL = control, Mon = monensin , GP= gas production , CH_4= methane, DM=dry matter, DOM=degraded organic matter, TDOM= truly degraded organic matter

3.3.3 Rumen fermentation parameters and degradability

There were no significant differences between the treatments and CTL in rumen NH_3-N and TDDM. The $BRP_{50\mu g}$ and $EBP_{25\mu g}$ improved (P<0.03) the TDOM, while MON decreased (P<0.03) the TDOM when compared with CTL. Monensin had the highest (P<0.002) value of PF compared to CTL (Table 3.3) while both types of propolis decreased (P<0.002) the protozoa counts compared with CTL.

The results showed that total short chain fatty acids increased significantly in all propolis treatments compared to CLT (Table 3.4). Both types of propolis (BRB and EBP) increased (P<0.001) acetic, propionic, butyric and acetic propionic ratio compared with CLT, while MON increased (P<0.01) propionic acid and decreased (P<0.001) acetic propionic ratio.

Table 3.3 - Effect of inclusion Brazilian Red Propolis (BRP) and Egyptian Brown propolis (EBP) on degradability and rumen fermentation *in vitro*

Additives	NH_3-N mg/100mL	TDDM g/kg	TDOM g/kg	PF DOMmg/ mL gas	Protozoa 10^5/mL
CTL	23.6	664.8	690.3^{b}	2.2^{b}	6.5^{a}
Mon	25.6	682.8	666.9^{c}	2.8^{a}	4.7^{ab}
$BRP_{25\ \mu g}$	24.4	701.2	715.2^{ab}	2.4^{ab}	3.5^{b}
$BRP_{50\ \mu g}$	25.7	708.0	721.2^{a}	2.5^{ab}	4.1^{b}
$BRP_{100\mu g}$	24.4	698.7	713.1^{ab}	2.4^{ab}	3.0^{b}
$EBP_{25\ \mu g}$	23.5	697.7	732.8^{a}	2.5^{ab}	3.3^{b}
$EBP_{50\ \mu g}$	25.1	693.8	708.3^{ab}	2.5^{ab}	2.9^{b}
$EBP_{100\ \mu g}$	24.7	679.2	701.8^{ab}	2.4^{ab}	3.7^{b}
SEM	2.61	67.41	54.01	0.48	3.24
*P*value	0.692	0.865	0.036	<0.001	0.002

SEM =stander error of the mean.

^{a,b,} means with different superscripts, within column, are different (Duncan test, P<0.05)

CTL =control, Mon = monensin, TDDM=truly degrade dry matter, TDOM= truly degraded organic matter, PF = partitioning factor

Table 3.4 - Effect of inclusion Brazilian Red Propolis (BRP) and Egyptian Brown propolis (EBP) on short chain fatty acids (SCFA) mM/L

Additives	Acetic	Propionic	Butyric	Isobutyric	Valeric	Isovaleric	A/P ratio	Total SCFA
CTL	41.80[b]	10.02[b]	8.54[b]	6.80	5.87	2.88	3.81[b]	75.94[c]
Mon	45.55[b]	13.81[a]	7.78[b]	5.49	6.96	2.56	3.28[c]	82.16 [bc]
BRP$_{25\ \mu g}$	59.39[a]	13.61[a]	10.82[a]	6.34	6.42	1.62	4.36[a]	98.20[ab]
BRP$_{50\ \mu g}$	58.56[a]	13.39[a]	10.46[a]	5.89	5.79	1.53	4.39[a]	95.63[ab]
BRP$_{100\ \mu g}$	60.42[a]	13.99[a]	10.94[a]	9.41	6.36	1.63	4.32[a]	102.76[a]
EBP$_{25\ \mu g}$	57.92[a]	13.54[a]	10.63[a]	6.31	6.21	1.62	4.28[a]	96.23[ab]
EBP$_{50\ \mu g}$	61.63[a]	14.05[a]	10.99[a]	3.13	6.21	1.59	4.39[a]	97.60[ab]
EBP$_{100\ \mu g}$	60.77[a]	13.88[a]	10.97[a]	6.32	6.41	1.62	4.38[a]	99.97[ab]
SEM	7.89	2.11	1.15	3.58	4.02	1.32	0.143	11.59
***P*.value**	0.007	0.015	<0.001	0.667	1.000	0.585	<0.001	0.032

SEM =stander error of the mean.

[a,b,] means with different superscripts, within column, are different (Duncan test, P<0.05)

3.4 Discussion

The Brazilian red propolis has been widely studied to elucidate its chemical composition and 12 types of Brazilian propolis have been characterized and classified into types 1–12 (PARK et al., 2000). The results of GC–MS analysis of the tested Brazilian propolis confirmed that this type was not included among these 12 types that classified by (PARK et al., 2000) and the current propolis was the type 13, which first classified by Alencar et al. (2007) since isoflavonoides (vestitol (24.62%) and medicarpin (13.52%)) were presented the most abundant compounds by the GC–MS analysis that never before reported in the previously 12 Brazilian propolis types (PARK et al., 2002; ALENCAR et al., 2007).

The Egyptian brown propolis (EBP) analysis showed high percentage of long chain fatty acids, such as palmitic acid (24.42%) and flavonoids as 3-(Hydroxymethyl)-1-Phenyl-1-Heptadecyn-3-Ol (13.75%) and flavone (10.15%) in EBP samples. This is in agreement with DUARTE et al. (2006) when presented the chemical composition of propolis type 6 and its bioactive hexane fraction revealed a high relative percentage of fatty acids, such as oleic, palmitic and stearic acids.

The differences of the major components of the Brazilin and Egyptian propolis confirmed the effect of the geographical location in the chemical composition consequently its biological activity is closely related to the vegetation native to the site of collection (PARK et al., 2002 and ALENCAR et al., 2007). The data suggest that these major compounds may be associated with the biological properties and main activity of these varieties of propolis since isoflavones, flavonoids and fatty acids have been reported as an antioxidant, antimicrobial, antiprotozoal and antifungal activity (CATTANI et al., 2012; ALENCAR et al., 2007; RUFER; KULLING, 2006).

In the present study, the antimethanogenic activity of MON (up to 44% CH_4 inhibition), which associated with the decreasing in GP was expected due to the lipophilic properties of MON against gram-positive ruminal bacterium, which produce more ammonia, hydrogen, and lactate than to gram negative bacteria, which produce succinic acid (CALSAMIGLIA et al., 2007). Monensin may have caused a decrease in intracellular K^+, a decrease in intracellular pH and an increase in intracellular Na^+ for gram positive bacteria (RUSSELL, 1987). As a result, decreased concentration of acetate, increased concentrate of propionate and reduced actetate: propionate ratio were consistent with the known mode of action of MON to decrease CH_4 production (RUSSELL; STROBEL, 1988).

Both BRP $_{50\mu g}$ and EBP$_{25\mu g}$ decreased significantly net CH_4 production (mL/g TDOM) by 26.4% for both. This inhibition could be due to the main constituents of each isoflavonoids and fatty acids. Park et al. (1998) suggested that main pharmacological action of propolis could be due to the hydrolysis of flavonoids to aglycone form free (PADMAVATI et al., 1997) that found to be more effective against gram-positive bacterial strains than on gram-negative (MIRZOEVA et al., 1997) in pure cultures.

According to Hayes; Berkovitz (1979), fatty acids act as anionic surfactants and have antibacterial and antifungal properties; these fatty acids can be selective against gram positive organisms by targeting the structure and function of bacterial cell walls and membranes. Iwami et al. (1995) suggested several mechanisms of the antibacterial activity of fatty acids, such as the inhibition of ATP regeneration and the maintenance of a pH gradient across the cell membrane of gram positive bacteria thus it could be characterizes propolis and its components as the ionophore monensin.

All propolis treatments showed significant decrease in protozoa count compared with CTL, which are in agreement with Rispoli et al. (2009) who found that propolis extract reduced the numbers of ciliates in the rumen of buffaloes fed a diet consisting of corn silage and concentrate (1:1) but not in cattle fed the same diet. This antiprotozoal effect could partially explain the reduction in CH_4 emission since the inhibition of protozoa reduces CH_4 release by diverting reducing equivalents from CH_4 to propionate synthesis in the rumen (KREUZER et al., 1986).

However, there were no significant differences in the mean values of PF either for MON or propolis treatments, which theoretically reflect similarity in their partitioning of the degraded organic matter to microbial mass (BLÜMMEL et al., 1999b) but MON showed significant differences in the TDOM and the SCFA than propolis. Mon decreased TDOM (Table 3.4) that could be partially explained by the sensibility of some cellulolytic bacteria for MON in the short-term *in vitro* experiments (RUSSELL; STROBEL, 1988) and decreased acetate:propionate ratio without effect on total SCFA compared with CTL (Table 3.5). In contrast, propolis presented different mode of action, since both (BRP$_{50\mu g}$ and EBP $_{25\mu g}$) resulted not only improving TDOM compared with both CTL and MON but also increased the acetate, propionate, butyrate and total SCFA. Our results are in agreement with (STRADIOTTI et al., 2004) who reported that propolis extract increased the total SCFA concentration in Holstein steers fed a diet containing 65% forage and 35% concentrate. While, Lana et al. (2007) showed that the supplementation of propolis (up to 6 g/animal/day) did not

affect total and individual SCFA concentrations in dairy goats fed a diet of 67% corn silage and 33% concentrate.

In the current study, the enhancement in the SCFA and TDOM by propolis addition suggested that the rumen fermentation process was improved. The improvement in the TDOM could be due partially to the degradation products of propolis flavonoids content that are capable of influencing fiber degradability since the bacterial ring fission of aglycone flavonoids produces phenolic acids, such as 3, 4-dihydroxyphenylacetic acid from isoquercitrin and quercetin or phenylacetic acid from naringenin (WINTER et al., 1989). Some of these simple phenolic compounds may interact with biosynthesis of aromatic amino acids, as both biosynthesis pathways are linked through cinnamic acid. Phenylpropanoic acid and phenylacetic acid have been reported to enhance cellulose degradation and growth of several strains of *Ruminococcus albus* (STACK; COTTA, 1986).

Also however, literature information regarding the effects of synthetic or natural antioxidant compounds on rumen fermentation and microbial growth is very scarce (CATTANI et al., 2012) but the antioxidant properties of the propolis major content (i.e flavonoids, propanoic acid and Medicarpin) may explain such improving in rumen fermentation process.

Studies *in vitro* found that some natural and synthetic antioxidants enhanced activity and growth of rumen microbes, promoting digestion and efficiency of diet utilization (VÁZQUEZ-AÑÓN; JENKINS, 2007). Because rumen microbes are predominantly strictly anaerobes with less developed antioxidant capacity than facultative anaerobic and aerobic microbes, it is possible that the addition of antioxidants would lessen oxidative stress, thus promoting better conditions for microbial growth (CATTANI et al., 2012). Smith et al. (2002) observed that the addition of 0.05 mg/g of ethoxyquin to dairy cow diets improved milk yield as well as DM digestibility, suggesting an antioxidant effect of this compound on rumen fermentation. However further investigation is needed to confirm this hypothesis.

The results of the current study suggested that propolis might be a useful feed additive for decreasing CH_4 production, enhance the rumen microbial fermentation and diet degradability. The same finding where mentioned by (ÍTAVO et al., 2011) who demonstrated that propolis extract can be potentially used as feed supplement instead of monensin sodium in the diets of feedlot lambs. Thus our study suggested the extraction of propolis major active compounds to be produced commercially may need to be taken into account.

3.5 Conclusions

The current study demonstrated that either $BRP_{50\mu g}$ and $EBP_{25\mu g}$ were reduced CH_4 emission as MON treatment but, the rumen TDOM was enhanced by propolis treatment compared with MON. Furthermore, propolis was capable of improving the rumen fermentation, increasing the individual and total SCFA and promising CH_4 mitigation agents in ruminants but more studies needed to confirm this effect *in vivo*.

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4 EFFECT OF BRAZILIAN RED PROPOLIS ADMINISTRATION ON REPRODUCTIVE PERFORMANCE, BLOOD VARIABLES PROFILE AND PARASITIC RESPONSE OF SANTA INÊS EWES DURING AND AFTER FLUSHING PERIOD

Abstract

The aim of this study was to evaluate the oral administration of Brazilian red propolis (BRP) extracted by ethanol 70% during and after flushing period on the reproductive performance and health responses of Santa inês ewes. Thirty adult grazing ewes aging 2.5 years and weighing 40 ± 1.8 kg live weight were grazing tropical grass pasture Brachiaria *decumbens*, elephant grass (*Pennisetum purpureum*) and supplemented with 4 % of body weight (BW) as flushing after estrus synchronizations. The substrate was a diet containing 50% Tifton (*Cynodon* spp) hay and 50% concentrate mixture Ewes were divided into control group (n=15) and propolis group (3 g of BRP / ewe/ day, n=15) administered orally. The treatment period was 21 day until the end of flushing period. Blood and fecal samples were collected weekly during and after flushing periods for 8 weeks. Blood samples were collected from the jugular vein in order to determine hormones, hematological and biochemical parameters. Pregnancy diagnosis was carried out using aid of transrectal ultrasonography. The results showed that BRP did not affect (P>0.05) on reproduction traits, but there were promising improvement on the number of services per conceptions (NS/C). Propolis was able to increase (P< 0.01) progesterone (P_4) concentration, and decreased (P< 0.05) cortisol (Cort), thyroxin (T_4), without significant changes in triiodothyronine (T_3) concentration. Mean values of BW and body condition score (BCS) were not significantly affected by BRP compared to the control group. Propolis resulted in increasing ($P < 0.01$) of total leukocyte (WBC) while, there were no differences observed for other hematological parameters. Results indicate that BRP increased ($P < 0.01$) total protein (TP) and globulin (G). On the other hand triglycerides (TG), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) decreased ($P < 0.01$) by propolis treatment. Propolis resulted in decrease ($P< 0.05$) of FEC compared to control. It can be concluded that BRP did not affect negatively on reproductive performance and it might act as an anti-stress agent. Propolis administration presented a good impact on the ewe's health including hematological, biochemical and parasitic response and it may be promising feed additive during a critical period such as flushing period.

Key words: Isoflavonoids. Propolis. Reproduction. Parasites. Serum parameters.

4.1 Introduction

There is increasing interest in exploiting natural additives as manipulators of rumen fermentation and have potentiality to improve feed efficiency, growth, immune response and over all sound health of the animals. Propolis is a resinous substance collected by honeybees from buds and leaves of trees and plants, mixing with pollen. Substances, which are identified in propolis, generally are typical constituents of food and/or food additives, and are recognized as generally recognized as Safe (GRAS) substances (BURDOCK, 1998). Numerous studies have proven its versatile pharmacological activities such as antibacterial, antifungal, antiviral, anti-inflammatory, hepatoprotective, antioxidant, antitumoral, and immunomodulatory (BANSKOTA et al., 2001; ALENCAR et al., 2007 ; SFORCIN, 2007). More than 300 constituents have been identified in different propolis samples (KHALIL, 2006). Propolis contains a variety of chemical compounds such as polyphenols (flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones), sesquiterpene quinones, coumarins, steroids, amino acids, and inorganic compounds (BANKOVA et al., 2000).

Propolis is an alternative to the use of dietary antibiotics because using antibiotics lead to the risk of transferring residues into meat and milk and resistant strains of bacteria. Thus, the use of antibiotics in animal nutrition has been prohibited in the European Union since January 2006 (OEZTUERK; SAGMANLIGIL, 2009). According to Mirzoeva et al. (1997), propolis has bacteriostatic activity against gram-positive and some gram-negative bacteria. The action mechanism of propolis is similar to the action of ionophores. Earlier studies reported that use of propolis widely in animal production and enhanced the performance and carcass quality of chickens subjected to heat stress (TATLI SEVEN et al., 2008).

Dietary addition of propolis was shown to enhance the performance and carcass quality of feedlot-finished bulls (ZAWADZKI et al., 2011). Propolis extract can be potentially used as feed supplement instead of monensin sodium in the diets of feedlot lambs (ÍTAVO et al., 2011). Moreover, the ethanol extract of propolis affects fermentation and methanogenesis of continuous microbial culture in ruminants. It decreases protozoa population and increases propionate levels by 10.3% (BRODISCOU et al., 2000). The inhibitory propolis action, *in vitro* and *in vivo*, on the deamination of amino acids was reported by Stradiotti Junior et al. (2004), which can mean greater ruminal protein escape, with consequent improvement of production efficiency of ruminants.

Despite all these advantages resulting from the propolis treatment, it may have deleterious effect on the animal reproduction such effect may be regarding to propolis major components of isoflavonoids, which structurally similar to steroid hormones, even though flavonoids are not cholesterol-derived compounds. However, hydroxyl in carbon 7 and in carbon 4′ have a similar disposition to that found in estradiol, allowing isoflavones to bind to estrogen receptor and activate it (D'ARCHIVIO et al., 2007) such as effect can inhibit hydroxysteroid dehydrogenase (HSDs) responsible for biosynthesis or degradation of steroid hormones, such as P_4. For example, various flavonoids and isoflavonoids such as apigenin is the most potent inhibitor of placental 17β-HSD. Furthermore, flavonoids are known to inhibit effectively 17β-HSD type 5, 3β-HSD type II and 11β-HSD (OHNO et al., 2004). However, we found very limited studies regarding to the effect of propolis extract on ewes reproduction performance and ewe's health including hormones, hematological, biochemical and parasitic response. Such as theses parameters of ewes are determined as an index of their health status as well. As a sign of stress specially at critical period as pre mating (flushing period) , the use of hormones, hematological, biochemical and parasitic response methods provides valuable knowledge about physiological reactions occurring against changing conditions, especially understanding the physiological, biochemical and hematological changes occurring at this period dealing with propolis treatment. Considering the above mentioned facts, to our knowledge there have been no investigations about this area of subject in ewes. Therefore, the present work attempted to address lack of information by describing the general action of propolis ethanolic extract administration on overall health of ewes through determining the reproduction performance and some hormones, hematological , biochemical and parasitic response of Santa inês ewes during and after flushing period.

4.2 Material and Methods

4.2.1. Origin and preparation of propolis extraction

Brazilian red propolis (BRP) sample was collected in January 2011 from the mangrove region in Marechal Deodoro city, Alagoas State, in Northeastern Brazil. This region is wet tropical climate (**9° 42′ 36″ S, 35° 53′ 42″ W)** and classified as type 13 The collected samples were weighted and stored separately in the refrigerator 4 °C until extraction. All chemical analysis of propolis was done at the Department of Agri-Food industry, Food and

Nutrition, “Luiz de Queiroz” College of Agriculture, University of São Paulo (USP), Piracicaba, SP, Brazil.

Propolis extraction was done according to Alencar et al. (2007) with some modification in the method, crude propolis sample (10g) was treated with liquid nitrogen and then grounded to a fine powder which mixed with 100 mL ethanol 70% then the mixture was transferred to ultrasonic equipment (Kerry, ultrasonic limited model, PUL,250, England) for 30 minutes. The ethanol extract solution was subsequently filtered through a filter paper (Whatman # 41) The extracted solution was incubated at the freezer (-5 °C) over night then it was filtered again, the supernatant was transferred to the rotary-evaporator at approximately 42 °C for 30 minutes in order to remove all the ethanol and the concentrated extract recovered in the volumetric flask was lyophilized for 3 days to get the resultant brownish resin (pure propolis)

4.2.2 Propolis analysis and chemical composition using Gas Chromatography–Mass Spectrometry (GC-MS)

The BRP sample was chemically analyzed after methylation of the extracts;About 5 mg of propolis was mixed with 75 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Sigma-Aldrich) in a sealed glass tube for 15 min at 60 °C to form TMS derivatives as described by Markham et al. (1996). Sample of the methylated solutions was analyzed by GC-MS according to (FERNÁNDEZ et al., 2008) with some modifications .A Shimadzu Model GC 2010 Series gas chromatograph coupled with an Shimadzu series mass-selective detector quadrupole mass spectrometer model GCMS-QP 2010 plus was used. Sample was separated on a 30 m × 0.25 mm i.d., 0.25 μm film thickness, capillary column (RTX5MS). The column temperature was initially held at 80 °C for 1 min, and then the temperature was raised to 320 °C at a rate of 5 °C min-1, followed by isothermal period of 20 min. Ultra-high-purity helium carrier gas at a flow rate of 1.0 mL/min. The injector was heated to 280 °C and was on split mode with a split ratio of 1:20, and the injection volume was 1.0 μL. The MSD was acquiring data in the full scan mode (mass range 40-800), with a multiplier voltage of 1000 V and ionization energy of 70 eV. The GC–MS peaks were identified by comparison with data from literature Piccinelli et al. (2005) and the profiles from the Nist 98 library, chromatograms of ethanolic extract and Identification of constituents of BRP was shown in Figure 4.3 and Table 4.2 respectively.

4.2.3 Experimental location and weather condition description

The laboratory analysis and *in vivo* experiment were done at the Laboratory of Animal Nutrition, Centre for Nuclear Energy in Agriculture CENA / USP (Piracicaba-Brazil) and all treatments and techniques used were in accordance to the Internal Commission for Environmental Ethics in Experimentation with Animals of the Centre for Nuclear Energy in Agriculture (University of São Paulo, São Paulo, Brazil). The daily mean ambient temperature and relative humidity throughout the experimental periods (1st of May to the end of July 2011) were obtained from a Meteorological Station of Piracicaba, Department of Bio Systems Engineering (LEB), Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo (USP). The weekly mean values of these parameters are shown in Figures 1.

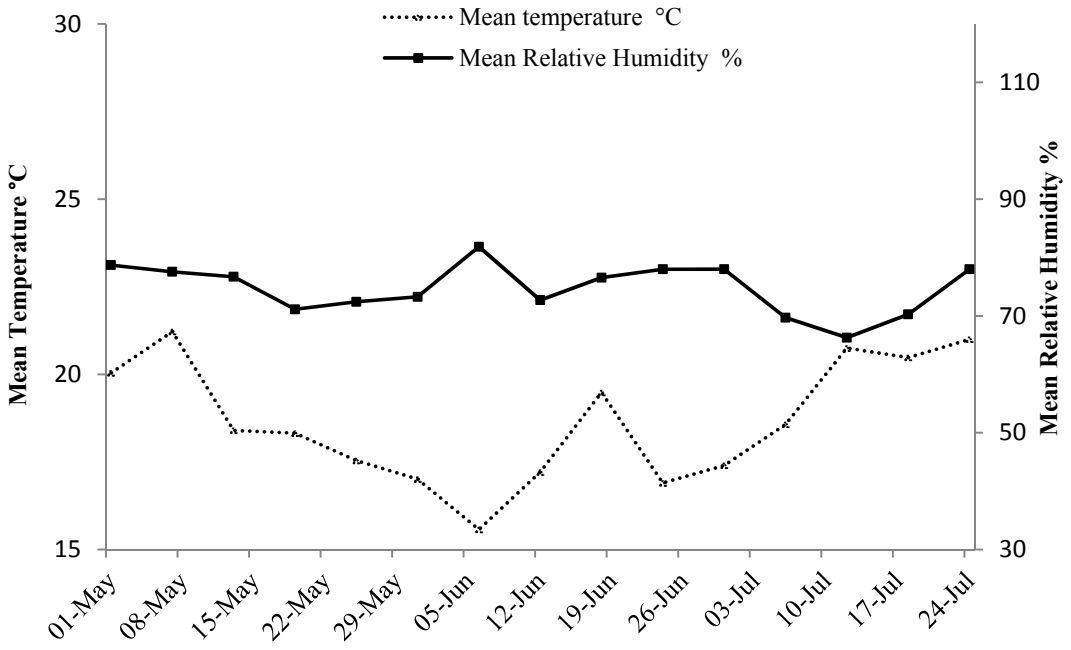

Figure 4.1 - Changes in mean ambient temperature (°C) and relative humidity (%) throughout the period from 1st of May to the end of July 2011

4.2.3.1 Animals and management

Thirty mature, non-lactating and non-pregnant Santa inês ewes aging 2.5 years and weighing between 40± 1.8 kg were used in this experiment. All animals were estrus synchronized before start flushing using double intramuscular injection of 75 µg/ head de-

cloprostenol (Ciosin®, Coopers, Brazil) with interval of 7 days (NOGUEIRA et al., 2009) as a source of $PGF_{2\alpha}$.

4.2.3.2 Animal flushing and propolis treatment

All ewes were grazing tropical grass pasture signal grass (*Brachiaria decumbens*), elephant grass (*Pennisetum purpureum*) and supplemented with 4% of BW as flushing after synchronizations. The experimental diet was composed of a fixed mixture composed of 50:50 (w/w) consisting of 50% of Tifton hay (Cynodon *spp.*) and 50% of concentrate (70% corn and 30% soya bean) and it was according to their body weight requirements (NRC, 2007). The diet was divided into two equal proportions to be offered two times in the day at early morning (8:00 AM) and after they back from the pasture (16:00 PM). Feed and chemical compositions of the experimental diet are summarized in Table 4.1. Water and mineral mixture were available ad libtum for all animals during the entire trial. Ewes were divided into two groups, 15 ewes each, according to their BW to control group and propolis group. At the same day of starting flushing the propolis group start to be administrated orally (3 g BRP /ewe /day) at early morning before access to their diet. The treatment lasted 21 day until the end of flushing period as shown in Figure 4.2. All animals were free of diseases and no behavioral abnormalities were detected throughout the experimental period. Animals were weighted weekly until the end of the experiment using digital scale.

Table 4.1 - Feed and chemical composition (g/kg on dry matter basis) of the experimental diet (fixed mixture of diet 50:50%)

Composition	g/kg
Feed composition	
Tifton 85 hay	500
Ground corn	337
Soybean meal	163
Chemical composition	
Dry matter	899
Organic matter	923
Crude protein	152
Ether extract	19
Neutral detergent fiber	548
Acid detergent fiber	256
Lignin	42

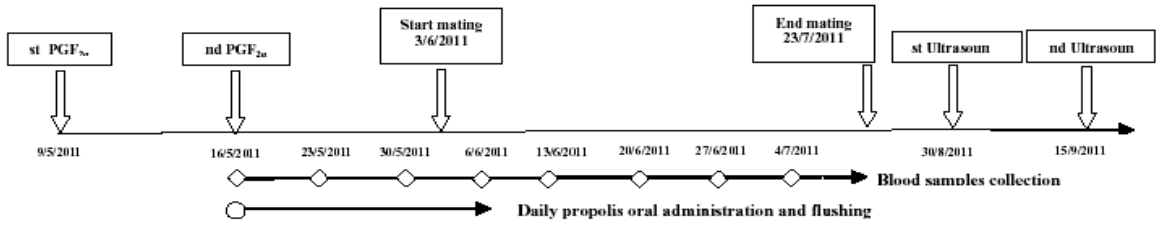

Figure 4.2 - Schedule of the experiment

4.2.4. Data collection

4.2.4.1 Estrus detection and Ultrasound diagnosis

Three fertile Santa inês rams were used to detect estrous behavior. Estrus observation was carried out twice daily at 6:00 to 8:00 AM and 18:00 to 20:00 PM during the mating period which extended for 51 days and approximately 3 estrus cycles as shown in Figure 4.2. After that, pregnancy diagnosis was carried out two times within 15 days in between using aid of transrectal ultrasonography. A real time B-mode scanner, equipped with (50 and 60 Hz) linear array probe, was used (Shenzhen mindray bio-medical electronics Co., LTD. Nanshan, China).

4.2.4.2 Reproductive performance

The reproductive performance in treated groups compared to the control group was evaluated using the following formulas according to ROSA et al.(2007); VATANKHAH et al. (2008); ALI et al. (2009).

a- Pregnancy rate (PR) = (no. of ewes pregnant / no. of all ewes in herd) × 100.
b- Fertility rate (FR) = (no. of ewes lambing / no. of ewes presented to rams) × 100.
c- Prolificacy rate or litter size (PRO) = (no. of lambs born / no. of ewes lambing) × 100.
d- Fecundity rate or lambing rate (FEC) = (no. of lambs born / no. of ewes presented to rams) × 100.
e- Number of services per conception (NS/C) = no. of services in all ewes / total number of conceived ewes.
f- Abortion rate (AR) = (no. of ewes aborting / no. of ewes pregnant) × 100.

4.2.4.3 Live body weight (BW) and body condition score (BCS)

Both BW and BCS for each ewe were recorded weekly throughout the experiment in the morning before access to feed and water. Body weight was done using a digital standing scale and body condition scoring (BCS = 1 for emaciated ewes to BCS = 5 for obese ewes at 0.5 intervals) has been described by Russel et al. (1969). The BCS of the ewes was taken by three trained people, who mastered the technique.

4.3.4.4 Blood samples and assays

Two blood samples were collected weekly from the jugular vein of each ewe during and after flushing periods as shown in Figure 4.2. Blood was collected in the morning, before access to feed and water and was placed immediately on ice. The first blood sample was collected using puncture into sterile glass flasks (5 mL), containing ethylenediamine tetra acetic acid (EDTA) as anticoagulant, whereas, the second sample was collected without using anticoagulant. The (EDTA) blood samples were used for determination of hematological parameters while the (none-EDTA) blood samples were centrifuged at 1500×*g* for 10 minutes to collect serum and were stored at -20 °C till hormones and biochemical analysis.

4.3.4.4.1 Serum hormones assay

All samples were analyzed, in duplicate, for concentrations of Progesterone (P_4), cortisol (Cort), triiodothyronine (T_3) and thyroxin (T_4) by using radioimmunoassay (RIA) for a commercial Siemens kit (Siemens Medical Solution Diagnostic, USA) using automatic Gama counter model (Wizard 2, Perkin Elmer, Dowens Grove, IL.USA). The sensitivities of assays were: 0.02 ng/mL (P_4), 0.2µg/dL (Cort), 7ng/dL (T_3) and 0.25µg/dL (T_4). The range of standards was from 0.1 to 40ng/mL, from 1 to 50µg/dL, from 20 to 600ng/dL and from 1 to 24µg/ dL in the P_4, Cort, T_3 and T_4 assays, respectively. For P_4 reference sera with mean concentration of 0.34 and 0.27 ng/mL, the intra- and interassay CV's were 8.8 and 9.7% or 13.2 and 14.8%, respectively. For Cort reference sera with mean concentration of 3.1 or 3.3µg/dL, the intra- and interassay coefficients of variation (CV's) were 4.8 and 5.2%, respectively. For T_3 reference sera with mean concentration of 56 and 59 ng/dL, the intra- and

interassay CV's were 8.9 and 10.0%, respectively and for T_4 reference sera with mean concentration of 2.4 and 2.3µg/dL, the intra- and interassay coefficients of variation (CV's) were 3.8 and 14.5% respectively.

4.3.4.4.2 Hematological parameters

The hematological analysis were determined on the same day after the blood samples were taken from ewes. Erythrocyte (RBC) were counted using new power chamber hemocytometer using a light microscope at 40 × 10 magnification. The blood samples were diluted 200 times by Hayem's reagent before counting (HEPLER, 1966). The concentration of blood hemoglobin (Hb) was detected by using the Sahli's method by colorimetric kits using a commercial hemoglobin standr (Ref 47, Labtest, Diagnóstica S.A®. Lagoa Santa, MG, Brasil). The Micro haematocrit tubes and haematocrit centrifuge were used to determine the packed cells volume (PCV) where blood was centrifuged for 5 min at 4000 rpm and the PCV value was obtained by reading the packed cells volume on the graduated hematocrit tubes. Total leucocyte counts (WBC) were detected using a new power chamber under a light microscope at 10 x 10 magnification after diluting the blood samples to 10 times with Turck's solution (Hepler, 1966).While other haematological parameters were calculated as fellow, mean corpuscular volume (MCV) = (PCV*10)/ RBC, mean corpuscular hemoglobin (MCH) = (Hb*10)/RBC and mean corpuscular hemoglobin concentration (MCHC) = (Hb *100)/PCV.

4.3.4.4.3 Serum Biochemical parameters

Blood serum concentrations of glucose (Gl), total protein (TP), albumin (A), triglycerides (TG), total cholesterol (Cho), oxaloacetic transferase (GOT), glutamate pyruvate transaminase (GPT), urea (UR), creatinin (CR), indirect bilirubin (IB) and total bilirubin (TB) were determined by colorimetric kits using a commercial Labtest (Diagnóstica S.A®. Lagoa Santa, MG, Brazil). Globulin (G) concentration was calculated as the difference between TP and A.

4.3.4.2 Faecal egg counts (FEC) analysis

The individual faecal samples were collected weekly as same as the protocol followed for collecting blood samples. To measure faecal egg counts (FEC) using the modified McMaster technique (UENO; GONÇALVES, 1994) for the period of eight weeks.

4.2.5 Statistical analysis

Data were analyzed by using PROC MIXED on SAS (2002).The model included treatment and week, as mean effect and their interaction, as follows:

$$Y_{ijk} = \mu + T_i + W_j + (TW)_{ij} + A_{kt} + e_{ijk}$$

Where:

Y_{ijk} = an observation taken on the k^{th} individual

μ = overall mean

T_i = a fixed effect of the i^{th} treatment (i=1 to 2)

W_j = a fixed effect of the j^{th} week (j=1 to 9)

$(TW)_{ij}$ = An interaction between i^{th} treatment and j^{th} week.

A_{kt} Random effect of the animal (inside treatment)

e_{ijk} = Random error assumed to be independent by and normally distributed with mean = 0 and variance = σ^2

The reproduction results were compared between groups using Chi-square. The FEC was transformed by log10 (x + 10) and differences at the 5% level were considered significant

4.3 Results

4.3.1 Propolis major constituents

The chromatograms of GC—MS of Brazilian red propolis (BRP) is shown in Figure 4.3 and Table 4.2. The relative percentage of the identified major compounds of the Brazilian propolis was isoflavonoides (42.5%), vestitol (24.62%), medicarpin (13.52%), isoliquiritigenin (2.46%) and formononetin (1.84%).

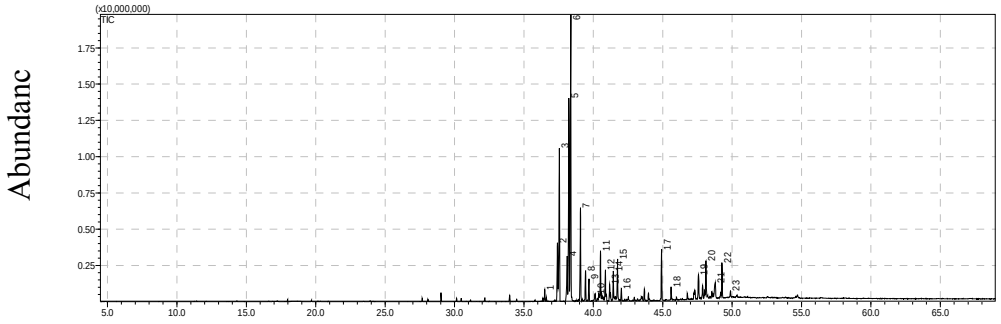

Figure 4.3 - GC-MS chromatograms of Brazilian red propolis (BRP)

Table 4.2 - Identification of Brazilian red propolis constituents by gas chromatography/mass spectrometry

Brazilian red propolis			
Peaks	Compounds	*t*R (min)*a*	(% area of each component)*e*
1	4,4' Bis[(Trimethylsilyl)Ethynyl]-2,2'-Bithiophene-5,5' Dicarbaldehyde	36.49	0.80
2	Silane, Trimethyl[5-Methyl-2-(1-Methylethyl)Phenoxy]	37.41	4.97
3	Medicarpin	37.55	13.52
4	Benzenepropanoic Acid, 3,4-Bis[(Trimethylsilyl)Oxy]-, Trimethylsilyl Ester	38.11	3.93
5	Benzenepropanoic Acid, 3,5-Bis[(Trimethylsilyl)Oxy]-, Trimethylsilyl Ester	38.24	16.49
6	Vestitol	38.37	24.62
7	4,4'-Bis[(Trimethylsilyl)Ethynyl]-2,2'-Bithiophene-5,5'-Dicarbaldehyde	39,07	7.07
8	Hydrocinnamic Acid, P-(Trimethylsiloxy)-, Trimethylsilyl Ester	39.43	1.96
9	3,4-Dihydroxy-9-Methoxypterocarpan	39.68	1.51

continue

				conclusion
	10	3,8-Dihydroxy-9-Methoxypterocarpan (3-Hydroxy-8,9-Dimethoxypterocarpan)	40.13	0.79
	11	9h-Fluorene-4,5-Diamine, N,N,N',N'-Tetramethyl	40.51	2.88
	12	Formononetin	40.86	1.84
	13	Silane, 9h-Fluoren-9-Ylidenebis(Trimethyl)	41.17	0.78
	14	2,5,?-Tri-Oh-Phenylacetate 4tms	41.44	1.77
	15	Isoliquiritigenin	41.74	2.64
	16	2-Propenoic Acid, 3-(3,4,5-Trimethoxyphenyl)-, Methyl Ester	42.02	0.70
	17	Silane, Trimethyl[1-[(Trimethylsilyl)Ethynyl]-2-Naphthalenyl]	44.91	3.30
	18	Propanedioic Acid, Bis[(Trimethylsilyl)Oxy]-, Bis(Trimethylsilyl) Ester	45.60	0.78
	19	Beta-Amirin Trimethylsilyl Ether	47.57	2.33
	20	Silane, (9,19-Cyclo-9.Beta.-Lanost-24-En-3.Beta.-Yloxy)Trimethyl	48.11	3.09
	21	9,19-Cyclolanostan-3-Ol, 24-Methylene-, (3.Beta.)	48.78	1.21
	22	Lup-20(29)-En-3-Yl Acetate	49.26	2.54
	23	Solanesol	49.88	0.48

*t*R (min)*a* = retention time , min)
*The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation

4.3.2 Effect of propolis on reproductive performance

The effect of oral administration of BRP on pregnancy rate, fertility rate, abortion rate, prolificacy rate, fecundity and number of services per conception are shown in Table 4.3. The reproductive performance including all these parameters in the treated group receiving BRP (3 g/ewe /day,) was not significantly (P>0.05) affected compared to the control group. Even it is of interest to note that ewes treated with BRP tended to enhance the NS/C.

Table 4.3. Reproductive performance of Santa inês ewes after administration of Brazilian red propolis (BRP)

Treatments	No. of ewes		No. of lambs born	PR%	FR%	AR%	PRO%	FEC%	NS/C
	Exposed	Lambed							
Control	15	13	15	86	80	7.69	125	100	1.77
Propolis	15	13	16	86	80	7.69	133	106	1.38
Pr > Chi Sq				1.00	1.00	1.00	0.96	0.96	0.85

Differences are not statistically significant. Pregnancy rate = PR, Fertility rate =FR, Abortion rate = AR, Prolificacy rate = PRO, Fecundity rate = FEC, Number of services per conception = NS/C.

4.3.3 Effect of propolis on BW and BCS.

The effect of BRP on body weight (BW) and body condition score (BCS) are shown in Figure 4.4. Mean values of BW and BCS were not affected (P>0.05) by BRP compared to the control group. However, BW and BCS were affected (P<0.05) by weeks throughout pregnancy period. Mean values of both parameters increased progressively throughout pregnancy period.

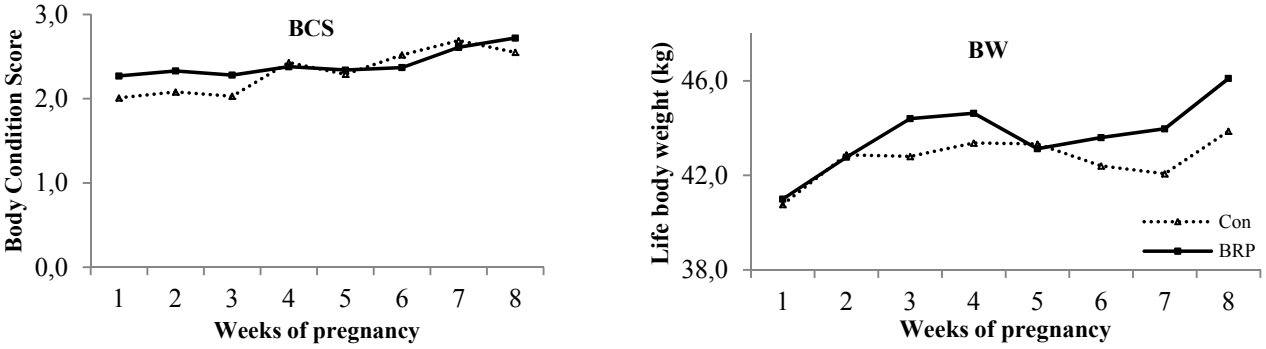

Figure 4.4 - Body weight (BW) and body condition score of Santa inês ewes after treaded with of Brazilian red propolis (BRP)

4.3.4 Effect of propolis on serum hormones

The effect of BRP supplemented on blood serum concentrations of P_4, cortisol, T_3 and T_4 are shown in Figures 4.5 and 4.6. Results showed that propolis increased ($P < 0.01$) P_4 and decreased cort concentrations compared to control group. Blood serum concentration of P_4 tended to increase (P<0.01) as pregnancy progressed while cortisol showed opposite trend.

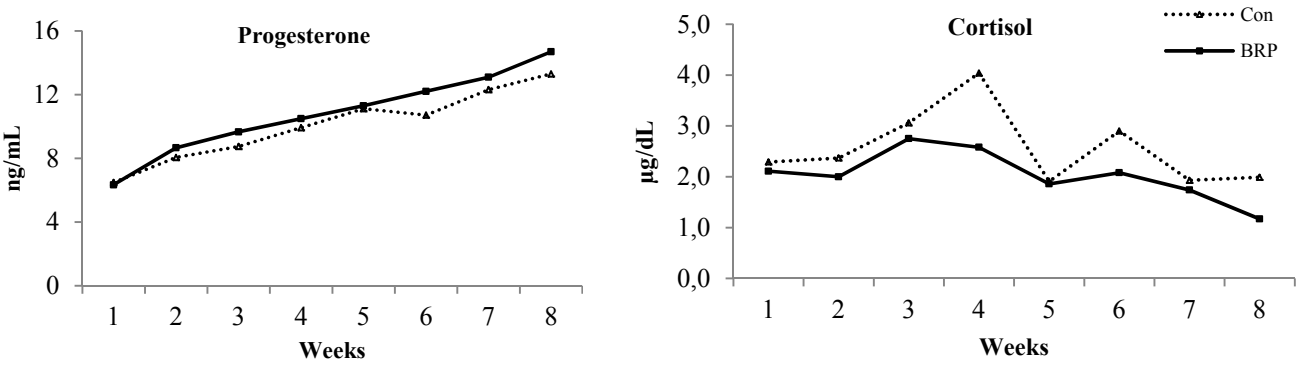

Figure 4.5 - The overall mean of progesterone (P_4) and cortisol concentrations of Santa inês ewes after Brazilian red propolis (BRP) supplementation

Triiodothyronine (T_3) did not changed (P>0.05) by BRP treatment but, T_4 concentration of the propolis treated group showed significant decreases (P<0.01) compared with control group. The overall mean of both concentrations of T_3 and T_4 tended to increase significantly by the advances of pregnancy.

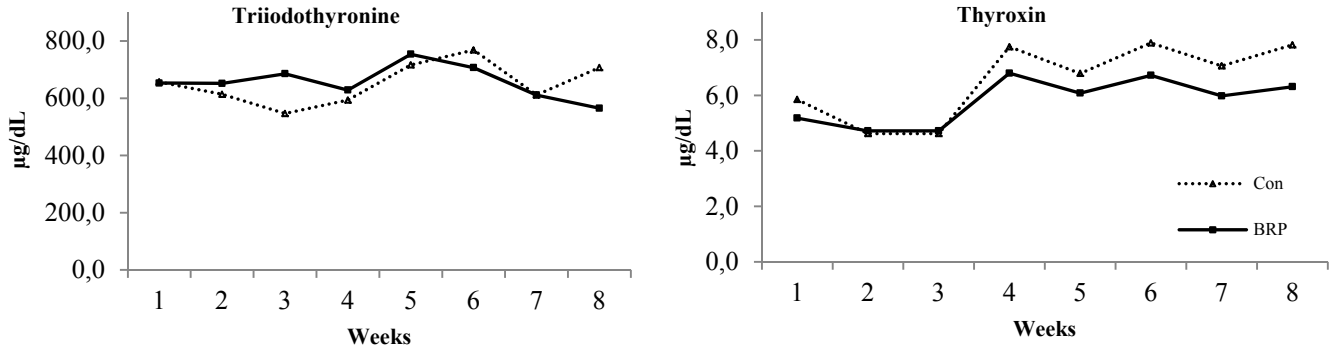

Figure 4.6 - The over all mean of triiodothyronine (T_3) and thyroxin (T_4) concentrations of Santa inês ewes after Brazilian red propolis (BRP) supplementation

4.3.5 Effect of propolis on haematological parameters.

The effect of BRP on haematological parameters and erythrocyte indexes are presented in Table 4.4. Propolis resulted in increasing ($P < 0.01$) of total leukocyte (WBC) compared with control, while there were no significant differences observed for other haematological parameters included (RBC, Hb, PCV, MCV, MCH and MCHC). Otherwise both Hb and PCV were not affected ($P > 0.05$) by the advances of weeks of pregnancy while, WBC and all erythrocyte indexes (MCV, MCH and MCHC) increased ($P < 0.01$) by pregnancy progress. Erythrocyte or red blood cells (RBC) decreased ($P < 0.01$) by pregnancy advanced. There is no significant effect observed among the treatment weeks interaction.

Table 4.4 - Haematological parameters and Erythrocyte indexes of Santa inês ewes after treaded with of Brazilian red propolis (BRP)

Haematological parameters	**Treatment**		**SEM**	***P*-value[a]**		
	Control	**Propolis**		**T**	**W**	**T x W**
Total leukocyte ($m^3/10^3$)	11.9[b]	13.1[a]	0.45	**	**	NS
Erythrocyte ($mm^3/10^6$)	13.1	13.0	0.22	NS	**	NS
Hemoglobin (g/dL)	11.5	11.3	0.18	NS	*	NS
Packed cell volume (%)	32.7	32.1	0.44	NS	*	NS
Erythrocyte indexes						
Mean corpuscular volume (μm^3)	25.4	25.3	0.39	NS	**	NS
Mean corpuscular hemoglobin (pg)	8.9	8.9	0.12	NS	**	NS
Mean corpuscular hemoglobin concentration (g/dl)	35.3	35.1	0.29	NS	*	NS

Data are mean values (n = 15), SEM= Stander error mean, NS= Not significant

[a] T = treatment effect, W = week effect, T×W = treatment week interaction ($P > 0.05$). Within a row, means with different letters (a and b) are significantly different *($P < 0.05$), ** ($P < 0.01$).

4.3.6 Effect of propolis on biochemical parameters.

Effect of BRP on blood serum concentrations of glucose, total protein (TP), albumin (A), globulin, triglycerides (TG), cholesterol (Cho), GOT, GPT, urea (UR), creatinine, and total bilirubin (TB) are presented in Table 4.5. Results indicated that BRP administration increased ($P < 0.01$) TP and globuline. On the other hand GOT and GPT decreased significantly ($P < 0.01$) by propolis treatment. Regarding to the effect of weeks the results indicated that all biochemical parameters were affected significantly ($P < 0.01$) by weeks but

in different manner except TB which did not affected significantly. glucose, TP, A, TG, Cho, Cr, GPT, UR and GOT were increased ($P < 0.01$) by the advances of pregnancy while, globulin was fluctuated by week's progress.

Table 4.5 - Serum biochemical parameters of Santa inês ewes after treaded with Brazilian red propolis (BRP)

Biochemical parameters	Treatment		SEM	*P*-value[a]		
	Control	Propolis		T	W	T x W
Glucose (mg/dL)	60.8	59.4	1.85	NS	**	*
Total protein(g/dL)	7.1[b]	7.6[a]	0.12	**	**	*
Albumin (g/dL)	2.8	2.8	0.05	NS	**	*
Globulin (g/dL)	4.4[b]	4.8[a]	0.11	**	**	**
Triglycerides (mg/dL)	21.4[a]	16.6[b]	0.91	**	**	*
Total cholesterol (mg/dL)	61.3	61.2	1.84	NS	**	NS
GOT (U/mL)	32.2[a]	29.7[b]	1.41	*	**	NS
GPT (U/L)	11.1[a]	9.9[b]	0.50	*	*	NS
Urea (mg/dL)	44.4	45.7	1.27	NS	**	NS
Creatinine (mg/dL)	0.57	0.54	0.02	NS	**	NS
Total bilirubin (mg/dL)	1.4	1.5	0.12	NS	NS	NS

Data are mean values (n = 15), SEM= Stander error mean, NS= Not significant

[a] T, treatment effect, W, week effect, T×W, treatment week interaction ($P > 0.05$). Within a row, means with different letters (a and b) are significantly different *($P < 0.05$), ** ($P < 0.01$).

4.3.7 Effect of propolis on fecal egg counts.

The effect of oral administration of BRP on the fecal egg count in Santa Inês ewes during and after flushing period are presented in Figure 4.7. Results showed that propolis resulted in decrease ($P< 0.05$) of FEC compared to control.

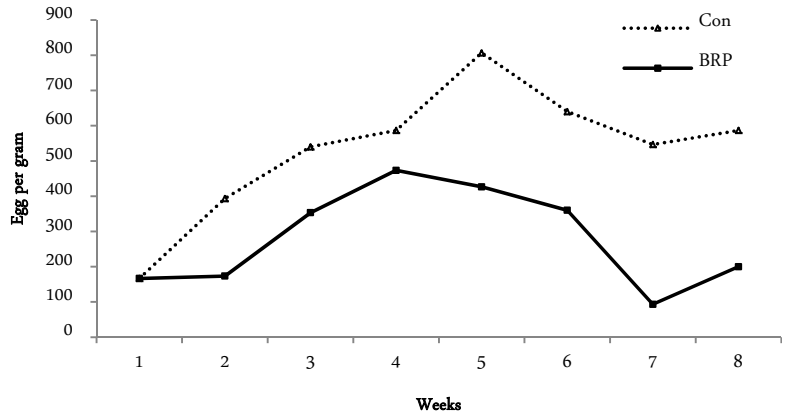

Figure 4.7 - Non-transformed mean eggs per gram of feces for Santa inês ewes after treaded with Brazilian red propolis (BRP)

4.4. Discussion

4.4.1. Propolis chemical composition and ewes' reproductive performanc.

The Brazilian red propolis has been widely studied to elucidate its chemical composition and 12 types of Brazilian propolis have been characterized and classified into types 1–12 (PARK et al., 2000). The results of GC–MS analysis of the tested Brazilian red propolis confirmed that this type was not included among these 12 types that classified by (PARK et al., 2000) and the current propolis was the type 13 (SILVA et al., 2008) which first classified by Silva et al. (2007) since isoflavonoides (medicarpin 13.52%, vestitol 24.62%, formononetin 1.82% and Isoliquiritigenin 2.64%) were presented the most abundant compounds by the GC–MS analysis that never before reported in the previously 12 Brazilian propolis types (PARK et al., 2002; ALENCAR et al., 2007). The data suggest that these major compounds may be associated with the biological properties and main activity of these varieties of propolis since isoflavonoides, have been reported as an antioxidant, antimicrobial, antiprotozoal and antifungal activity (CATTANI et al., 2012; ALENCAR et al., 2007).

Isoflavonoids presented the major components in BRP and these flavonoids can act as hormones in both plants and animals (BAKER, 1998). The estrogenic effect of some

flavonoids originally was discovered by observing the behavior of sheep that had eaten fermented clover became sexually aroused (SONNENBICHLER et al., 1980). Since estrogens also have anabolic effects (SOKOLOVA et al., 1978), one might suspect that flavonoids might be able to act as growth hormones in animals also. However, so far only few indications of such a function have been found (HAVSTEEN, 2002).

Flavonoids able to display an estrogenic and pregnancy inhibitory function (JAIN et al., 1993). This effect was already known by lay practitioners in the Middle Ages, but it now has been confirmed using modern methods. After ovariectomy, endothelial dysfunction resulting from the lack of estrogen can be improved by the supplementation with either genistein or 17-β-estradiol (SQUADRITO et al., 2000). The authors concluded that the effects of the two substances were overlapping. However, the discussion of the phyto-estrogenic effect of flavonoids in the literature has been rather controversial, especially regarding possible replacement therapy with genistein and estrogen to improve endothelial dysfunction (SQUADRITO et al., 2000). In our study the results showed that there were no negative effect was observed in the reproduction performance of ewes. Even there was enhancement of BRP to decrease the NS/C.

4.4.2. Effect of propolis on ewes BW and BCS

There were no significant effects of BRP on both BW and BCS Figure 4.4 at the current study, which in agreement with Ítavo et al. (2011) who demonstrated that, propolis (green and brown propolis) did not affected significantly on lambs BW. Even in the same study the authors mentioned that, flavonoides and phenol levels were higher in green propolis than in brown propolis extract. As a consequence, the higher concentration of these compounds in animal feed possibly exerted bactericidal action in lamb rumen, thereby explaining the poor feed conversion value but without affecting on final BW of lambs. The same finding was observed for post weaning Hanwoo calves by Sarker and Yang (2010). On the other hand, Zawadzki et al. (2011) concluded that, the addition of propolis extract to the diet of feedlot-finished bulls increased ($P < 0.03$) BW and improved feed conversions compared with sodium monensin.

The gradual increase in BW and BCS with the advance of pregnancy has been reported by many investigators in several animal species and was attributed to the growth of the fetus as well as to the accumulation of fetal fluids (ROBINSON et al., 1977). Additionally,

pregnancy in sheep and probably in most other species is accompanied by an increase in total body water and blood volume (ROBINSON et al., 1978) which contribute to the increase in body weight during pregnancy.

4.4.3. Propolis and ewes serum hormones

The explanation of increasing P_4 concentration by administration BRP (Figure 4.5), may be related the content of isoflavonoids, where these compounds have been shown to have extensive biological effects, such as enzyme inhibition and antioxidant activity. Because flavonoids are structurally similar to steroid hormones, they can inhibit hydroxysteroid dehydrogenase (HSDs) responsible for biosynthesis or degradation of steroid hormones, such as P_4. For example, of various flavonoids, apigenin is the most potent inhibitor of human placental 17β-HSD. Furthermore, flavonoids are known to inhibit effectively 17β-HSD type 5, 3β-HSD type II and 11β-HSD (OHNO et al., 2004).

The dramatic increase in P_4 secretion with the advance of pregnancy in ewes had been demonstrated by several studies (MANALU; SUMARYADI, 1998) who mentioned that P_4 concentration tend to increase by advances of fetal growth. It was revealed that P_4 concentration in sheep plasma during the first trimester of pregnancy (up to day 50) are comparable of those found during the luteal phase of the estrous cycle (BAZER; FIRST, 1983) suggesting that the corpus luteum is the main source of P_4 for pregnancy maintenance during this period.

Regarding to the decreasing cortisol concentration by propolis, it may be related to high concentration of flavonoieds. Our finding is in agreement with Ohno et al. (2002) who showed that flavonoieds were able to inhibit significantly cortisol secretion from H295R cells at concentrations of 12.5μM. and this may be related to the hydroxy group at position 6 of the pyran ring or the 4` position of the benzene ring of flavones seems to effect inhibition of cortisol production. In isoflavones, a hydroxy or methoxy group in the 4` position of the benzene ring effects inhibition of cortisol production, but the glucosyl group in the 7 position on the pyran ring does not. The decreasing level of cortisol after week 4 it may be related to the ending of oral treatment of propolis which it may present a type of stress for ewes where, since stress is relatively correlated with increasing cortisol concentration in the blood (RUSSELL et al., 2012).

Oral administration of BRP resulted in decreasing T_4, without changes in T_3 (Figure 4.6). This effect could be due to inhibition of 5′-deiodinase activity which may be related to propolis flavonoids content (SCHRODER-VAN DER ELST et al., 1991). Flavonoids are capable to interfere with thyroid hormone economy (GAITAN, 1996). Our results are consistent partially with Chandra and De (2010) who showed that flavonoieds are able to inhibit thyroperoxidase activity and reduce both serum T_3 and T_4 levels, and increase TSH concentration. In another study of the same group, Chandra et al. (2011), showed that green and black tea flavonoids extracts alter the thyroid gland physiology and architecture, with enlargement of thyroid gland as well as hypertrophy and/or hyperplasia of the thyroid follicles and inhibition of the thyroid peroxidase activity and type I 50-deiodinase. Thyroidal Na+, K+-ATPase activity was elevated, along with decrease in serum T_3 and T_4, and an increase in TSH level.

Additionally, results of the study by Sartelet et al. (1996) demonstrated potent antithyroid effects of two flavonoids present in Fonio millet (Digitaria exilis), showing that the prevalence of goiter may be influenced not only by iodine deficiency, but also by other nutritional factors. They showed that high amount of flavonoids capable to reduce both iodide organification and secretion of thyroid hormones. Thus, flavonoids may affect thyroid hormone economy at diverse levels, not only inhibiting iodide uptake and thyroid hormone biosynthesis but also interfering with T_4 to T_3 conversion and with the binding of T_4 to plasma protein. On the other hand, Bitto et al. (2010) have shown that the intake of genistein, an isoflavone present in soy foods did not significantly affect thyroid function. The same was observed in study by Bruce et al. (2003).

The rise in thyroidal activity for both hormones (T_3 and T_4) at early gestation may be due to the influence of the newly synthesized placental estrogens (AVRUSKIN et al., 1976). In addition the increasing concentrations of (T_3 and T_4) demonstrated a direct relationship with the nutritional level that have been confirmed by others researchers (NOZIÉRE et al., 2000; DELAVAUD et al., 2002) while all this animals were overfed as flushing so it may be a second reason for rising thyroidal activity at early gestation .

4.4.4. Propolis and ewes hematological parameters

In this study, the effects of Brazilian red propolis on hematological and some biochemical parameters were investigated. In the present study, the data showed significant ($P < 0.01$) increase for WBC counts in propolis group compared with control as shown in Table 4.4, which agree with those of Orsolić and Basic (2003; 2005) who reported that water-soluble derivatives of propolis (WSDP) given to mice caused a significant elevation of leucocytes in peripheral blood. The increase observed in total leukocyte count may indicate an activation of the animal's immune system. Previous studies have shown that propolis has anti-inflammatory and immunomodulatory activities (DE CASTRO, 2001; DANTAS et al., 2006). It has been also reported that propolis treatment increased proliferation of leucocyte precursors from pluripotent stem cell in mice (ORSOLIĆ; BASIC, 2005).Such as effect of BRP on WBC count may be related to the major active components' of propolis which are flavonoids. Many studies have been reported that, Stimulation of the immune system by flavonoids (HAVSTEEN, 2007). Propolis can act as animmunostimulant. Ample stimulation of the immune system of propolis can increase the total leukocytes value (TALAS; GULHAN, 2009).

Leucocyte counts were increased significantly during week of pregnancy. This is similar to that reported by Jain (1993), in that leucocyte numbers gradually increase during gestation to the day of parturition. These counts are usually above the normal values in most domestic animals (JAIN, 1993). On the other hand RBC counts tended to decreased significantly by pregnancy advances without any effect on both Hb and PCV. Such as effect may be explain the increasing value of (MCV, MCH and MCHC) by pregnancy progress. Similar results have been observed in pregnant sheep, mares and sows and bitches (JAIN, 1993; VIHAN; RAI, 1987). These results during the advances stages of gestation could be attributed to the hemodilution-effect, resulting from an increase in plasma levels of RBC's. These findings have also been observed in pregnant does (AZAB; ABDEL-MAKSOUD, 1999). The hemodilution of the domestic animals may be of physiological importance, as it decreases the blood flow in the capillary vessels and it may improve the blood flow through the placental capillary vessels - especially to increase the diffusion of O_2 and nutritients to the fetus (YÝLMAZ, 2000). This hypothesis has been confirmed by Pere et al. (1996).

4.4.5. Propolis and ewes serum biochemical parameters

In the present study, propolis increased significantly ($P < 0.01$) total protein concentration as shown in Table 4.5, the same finding was observed by Eraslan et al. (2007) who demonstrated that significant increase-compared to control group-was found in total protein values of the rat group which they gave propolis at 200 mg/kg BW) with drinking water for 21 days. Cetin et al. (2010) in his study on rats demonstrated that treatment of rats with propetamphos plus propolis increased total protein levels compared to the rats treated with propetamphos. This suggests that propolis can modulate protein metabolism.

Globulin concentration was highly significant ($P < 0.01$) in ewes treated with propolis, such increment could be attributed to stimulation of the immune system by propolis flavonoids (HAVSTEEN, 2002) or because the increasing concentration of TP which occur without affecting on A, where globulin was calculated as the difference between TP and A. On the other hand Sarker and Yang (2010) reported that propolis did not affect significantly on G concentration of post-weaning Hanowoo calves.

Our study indicated that treatment of ewes with propolis decreased triglyceride, compared to the control group. Decrease in triglyceride and levels may be concluded to be directly related to the influence of propolis on lipid metabolism. These findings are similar to the data reported by (CETIN et al., 2010). Similar results were obtained by Fuliang et al. (2005), and these researchers have reported propolis to cause decrease in triglyceride levels when administered to rats with diabetes mellitus. In addition, Kolankaya et al. (2002) reported that propolis caused a decrease in triglyceride level of rats treated with alcohol.

Among other biochemical parameters, the increase in GOT and GPT activities were found to be related to damage in the liver and the change in hepatic functions. In the present study, we observed a significant decrease ($P < 0.01$) in both GOT and GPT data presented in Table 4.5. This decrease supports the hepatoprotective effect of propolis. Similar results were obtained by Sugimoto et al. (1999), and these researchers have reported propolis to cause decrease in GOT activity when administered to rats exposed to D-galactosamine. Similarly, Chopra et al. (1995) have reported propolis to cause decrease in GOT activity that has increased due to exposure to doxorubicin in rats. Çetin et al. (2010) in his study to investigate the effectiveness of propolis in alleviating the toxicity of propetamphos on haematological and biochemical parameters in rats concluded that indicated that treatment with propetamphos alone increased the activities of GPT and GOT in serum. After treatment of rats with propetamphos plus propolis the activities of GOT and GPT were normalized to their control

values. This decrease supports the hepatoprotective effects of propolis. Flavonoids found in propolis may be responsible for the positive effect of propolis on these enzymes (SANZ et al., 1994). This result is in agreement with the findings that propolis induced reduction of the increased activity of GOT and GPT in plasma of rats treated with aluminium chloride (AL-SAYEDA et al., 2009) and alcohol (KOLANKAYA et al., 2002). This difference in our study is considered to may arise from the origin of propolis and its constituents. Furthermore, the duration of exposure, the dose exposed to and the physiological differences of the exposed animal may also lead to this result.

Regarding to the effect of weeks of pregnancy on these parameters, pregnancy period was found to have significant effect on all parameters under study Table 4.5. Blood parameters increased gradually by the advances of pregnancy, these results were in complete agreement with that of El-Sherif and Assad (2001) who demonstrated that, in dry ewes blood parameters remained constant during the experimental period, while in pregnant counterparts, these parameters increased gradually to reach their maximum level before parturition. The same finding was observed by Balıkc et al. (2007). Such as changes which occur in blood parameters could be explained by requirements of the ewe increase during pregnancy due to rapid growth of the fetus (FIRAT; ÖZPINAR, 2002).

4.4.6. Propolis and ewes FEC

Ewes treated with BRP showed significant decrease of FEC Figure 4.7. Our results with agreement with Principal et al. (2002). Who demonstrated that efficacy of propolis to control sheep helminthiasis and decrease significantly FEC in treated group compared with control. Moreover, Heinzen et al. (2012) concluded that the anthelmintic effect of alcoholic extract of propolis, at 30% of concentration, in naturally infected calves where 83% of animals showed an average decrease of 48.48% of FEC specially (*Trichostrongylus* sp. and *Strongyloides* sp). Principal et al. (2002) tested several levels of propolis extracts to control of helminthiasis in West African sheep and found that dose of 10 mL of an 3% ethanolic extracts of propolis was the most effective in this species. Araújo et al. (2006); Castagnara et al. (2007) found a reduction of FEC in Santa sheep breed, by using the 30% extract of propolis. Loureiro (2007) found that the addition of 30 mg of propolis extract to the diet of sheep was effective in reduce the number of FEC, demonstrating its effect antiparasitic and the possibility of its use in control of worms. Dürrewald et al. (2008) used alcoholic extract of propolis to 33% in

20 cattle twelve months naturally infected with *Trichuris* sp., *Tricostrongilos* sp. and *Ascaris* sp. administered in single dose once or twice a day for three days consecutive, and 30 days after the start of treatment, found an average reduction of 59.7% in the count FEC between animals receiving treatment, and average increase of 63.6% in the control animals that did not receive any treatment.

Propolis possesses variable biological activities: It had been proven to be 100% effective against some parasites such as lethal protozoa and would also decrease inflammation associated with parasite infection (HIGASHI; DE CASTRO, 1994). Previous study proved evidence of inhibitory activity of propolis on the vitality and hatchability of immature *Fasciola gigantica* eggs (HEGAZI et al., 2006). Fahmy et al. (2010) in his study on propolis observed that, animal group which given propolis revealed significant decrease ($P < 0.05$) in parasitological parameters, where percentage worm reduction was 68% for hepatic worms and intestinal eggs reduction being 68.0% and 70.60% respectively.

Moura et al. (1998) in his study on naturally infected New Zealand White growing rabbits by *Eimeria* spp and treated with hydroalcoholic propolis solution (HPS) (0.0; 4.0; 8.0; 12.0 and 16, 0 ml of HPS/L of water), observed that, addition of HPS levels to the drinking water showed a linear decline of the oocists *Eimeria* spp in drops of the rabbits faces. Furthermore, Hegazi et al. (2007) in his study on *Fasciola gigantica* concluded that, an alternative and effective fasciolicidal drug can be developed using propolis extract because propolis was high efficacy against both mature and immature flukes. Such as reduction of FEC which occur in our study could be attributed to propolis Flavonoids, hence Flavonoids were the main components of red propolis. In addition, flavonoids showed large antiparasitic activity (SALEM et al., 2011). Other explanation for decreasing FEC could be related to the stimulation of the immune system by propolis which reflected as increasing the total leukocytes value (TALAS; GULHAN, 2009).

4.5. Conclusions

Brazilian red propolis main constituents are isoflavonoids and oral administration of such natural product does not affect negatively on reproductive performance of Santa inês ewes and it might act as an anti-stress agent. Propolis had a good impact on ewes health and propolis can be used as promising feed additive during a critical period such as flushing period.

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5 EFFECT OF BRAZILIAN RED PROPOLIS SUPPLEMENTATION ON MILK YIELD, COMPOSITION AND LAMBS PERFORMANCE OF SANTA INÊS EWES

Abstract

The objective of this study was to evaluate the effect of oral administration of Brazilian red propolis (BRP) on milk yield, composition of *Santa inês ewes* and there lambs performance. Twenty late pregnant Santa inês ewes (55.5 ± 2.0 kg body weight; 2.8 ± 0.05 BCS) were used in this study from 25 ± 3 d prepartum through 48 d postpartum. Ewes were divided into two equal groups according to the body weight and parity, control (n=10) and propolis group (n=10). The propolis group received orally3 g propolis ethanolic extract /ewe/day at early morning before access to their diet for 21 days. Milk production was measured manually twice weekly postpartum for seven weeks. Ewes body weight (BW) and body condition score (BCS) for ewes were recorded weekly. Birth weight of each lamb was recorded after 24 h after birth in, weaning weight (WW) was recorded when lambs reached their eighth weeks of age. The results showed that, propolis administration increased ($P < 0.05$) milk yield, milk fat%, protein yield, fat yield, lactose yield and energy corrected milk. Somatic cell counts (SCC) were decreased ($P < 0.05$) by propolis treatment while, there were no significant ($P > 0.05$) deference were observed for other milk protein %, lactose % and pH. Propolis administration did not affect (P >0.05) on ewes BW but increased ($P < 0.05$) BCS. No differences were observed for lamb's birth weight and weaning weight but propolis increased ($P < 0.05$) average daily gain (ADG) and milk conversion ratio (MCR). Propolis extraction enhanced milk yield and milk quality which in turn affect positively on lambs performance.

Key words: Propolis. Milk yield. Somatic cell counts. Lambs performance.

5.1 Introduction

The use of additives in the diet of ruminants aims to improve production rate and feed conversion, may also act to promote greater stabilizing in the rumen environment (FERNANDES et al. 2008). Among the substances used to manipulate ruminal conditions are ionophores that are substances capable of acting on rumen bacteria by modifying the fermentation process (VAN SOEST, 1994). These substances are highly effective against gram-positive bacteria, no have effect on gram-negative, which have membranes capable of preventing their entry into cells (DUFFIELD; BAGG, 2000). More than 120 ionophores exist, only a few (lasalocid, monensin, salinomycin and propionate laidomicina) are authorized for use in ruminants diets (NAGAJARA et al., 1997). Using ionophores in ruminants diets presented good impact on rumen fermentation and animal productive performance (GUAN et al., 2006; OLIVEIRA et al., 2007) However, in recent years, this practice has faced reduced social acceptance in many countries, on grounds of meat and milk product quality and safety because use of antibiotics in livestock production increases prevalence of resistant bacteria and residual in meat and milk (MATHEW et al., 2001). Accordingly, many countries prohibit the use of antibiotics in raising livestock and restrict the importation of products derived from antibiotic-treated animals since January 2006 (OEZTUERK; SAGMANLIGIL, 2009). As a consequence, several researches have been done in order to discover alternative feed additives, which being natural and accepted by consumers like propolis.

Propolis is an alternative to the use of dietary antibiotics (ÍTAVO et al., 2011). According to Mirzoeva et al. (1997), propolis has bacteriostatic activity against gram-positive and some gram-negative bacteria. The action mechanism of propolis is likely related to changes in the bioenergetic status of the bacterial membrane, which inhibits bacterial motility. This is similar to the action of ionophores. Propolis is a resinous substance collected by honeybees from buds and leaves of trees and plants, mixing with pollen Substances, which are identified in propolis, generally are typical constituents of food and/or food additives, and are recognized as Generally Recognized As Safe (GRAS) substances (BURDOCK, 1998). Numerous studies have proven its versatile pharmacological activities: antibacterial, antifungal, antiviral, anti-inflammatory, hepatoprotective, antioxidant, antitumoral, etc. (BANSKOTA et al., 2001). Earlier studies reported that the use of propolis in animal production. For instance, the addition of the ethanoic extract of propolis had significant effect ($P<0.05$) on milk production and protein yield (FREITAS et al., 2009). Moreover, The antimicrobial activity of propolis ethanolic extract against the major microorganisms that

causing mastitis was observed *in vitro* as well as *in vivo* even in ovine or bovine species (LOGUERCIO et al., 2006; SILVA et al., 2012).

Supplementation of lactating West African goats diet in the tropical environment with different levels of Bee Wax Residue Meal which content propolis resulted in significant ($p<0.05$) increase of milk yield also increased butter fat, protein, calcium, phosphorus potassium, sodium and iron contents in traded animals compared with control (ADEWALE et al., 2010). Considering the above mentioned facts, this study evaluated the effects of oral administration of Brazilian red propolis on milk production, composition and lambs performance

5.2 Material and Methods

5.2.1 Propolis characterization

All chemical analysis of propolis and milk composition were done at the Department of Agri-Food industry, Food and Nutrition, and Department of Animal Science, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo(USP), Piracicaba, SP, Brazil.

Crude Brazilian red propolis (BRP) samples was collected in January 2011 from the mangrove region in Marechal Deodoro, a city in the vicinity of Maceio, capital of Alagoas State, in Northeastern Brazil, and classified as type 13 according to Park et al. (1998). The collected samples were weighed and stored separately in the refrigerator at 4°C until processing. In order to obtain propolis extracts, propolis extraction was done according to Alencar et al. (2007) with some modifications in the method. Crude propolis sample (10g) was treated with liquid nitrogen and then grounded to a fine powder, which mixed with 100 mL of ethanol 70%. Thehe mixture was transferred to ultrasonic equipment (Kerry, ultrasonic limited model, PUL, 250, England) for 30 minutes. The ethanol extract solution was subsequently filtered through a filter paper (Whatman # 41). The extracted solution was incubated at the freezer (-5 °C) over night then it was filtered again andthe supernatant was transferred to the rotary-evaporator at approximately 42°C for 30 minutes to remove the ethanol. The concentrated extract recovered in the volumetric flask was lyophilized for 3 days to get the resultant brownish resin (pure propolis). The BRP sample was chemically analyzed after methylation of the extracts by Gas Chromatography–Mass Spectrometry GC-MS according to Fernández et al. (2008).The GC–MS peaks were identified by comparison with

data base library (PICCINELLI et al., 2005) and the chemical profiles of BRP is shown in Table 1.

5.2.2 Experimental description

5.2.2.1 Local and ambient conditions of the experiment

The laboratory analysis and *in vivo* experimental were done at the Laboratory of Animal Nutrition, Centre for Nuclear Energy in Agriculture CENA / USP, Piracicaba **(**Brazil. The treatments and techniques used were in accordance to the Internal Commission for Environmental Ethics in Experimentation with Animals of the Centre for Nuclear Energy in Agriculture (University of São Paulo, São Paulo, Brazil). The daily mean ambient temperature and relative humidity throughout the experimental periods (10th October to the end of December 2011) were obtained from a Meteorological Station of Piracicaba, Department of Bio Systems Engineering (LEB), Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo (USP).The weekly mean values of these parameters are presented in Figures 5.1.

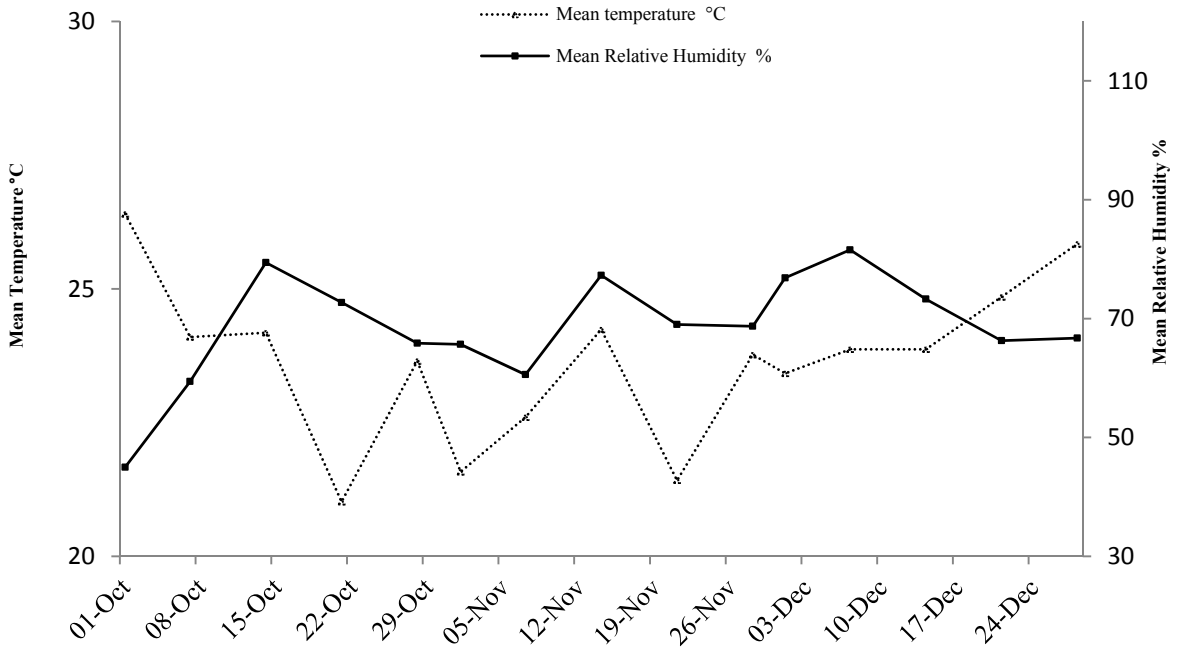

Figure 5.1 - Changes in mean ambient temperature (°C) and relative humidity (%) throughout the period from 10th of October to the end of December (2011)

5.2.2.2 Experimental design and treatment

Twenty late pregnant Santa inês ewes (55.5 ± 2.0 kg body weight; 2.8 ± 0.05 BCS) were used in a completely randomized design study from 25 ± 3 d prepartum through 48 d postpartum. The pregnancy days were estimated according to the time-frame of ram introduction into different ewe plots during the earlier breeding season. Ewes were pregnancy diagnosed from mating until pregnancy in order to confirming the expected date of schedule delivery using transrectal ultrasonography a real time B-mode scanner, equipped with (50 and 60 Hz) linear array probe, (Shenzhen mindray bio-medical electronics Co., LTD. Nanshan, China). Ewes were grazing on tropical grass pasture composed from signal grass (*Brachiaria decumbens*) and elephant grass (*Pennisetum purpureum*) and supplemented with total mixed rations (TMR) diet (4% of BW). The experimental diet was composed of a fixed mixture and composed of 50:50 (w/w) consisting of 50% of Tifton hay (Cynodon. *spp.*) and 50% of concentrate (70% corn and 30% soya bean) ingredients. Rations were mixed twice monthly and daily and allowances were offered for all animals

The diet was divided into two equal proportions to be offered two times in the day at early morning 08:00 h before pasture and at 17:00 h after pasture. Diets were sampled every 3 weeks upon mixing for chemical analysis and kept at 4 °C until analysis. Feed samples were ground by Wiley mill through a 1-mm screen and analyzed for dry matter (DM), organic matter (OM), crude protein (CP), and ether extract (EE) (AOAC, 2006). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to VAN SOEST et al.(1991). Ingredients and chemical composition of the experimental diet are summarized in Table 5.1. Water and vitamins mineral mixture were available adlibitum for all animals during the entire trial. Ewes were divided into two control and propolis groups and body weight and parity were considered in randomization. Each treated group had 10 ewes including three twin-lamb ewes and seven single-lamb ewes. Ewes at the propolis group administrated orally 3.0 g propolis ethanolic extract /day/ewe at early morning before access to their diet for 21 days. After treatment period, ewes were returned to feed the basal diet according to their requirements (NRC, 2007). All animals were free of diseases and no behavioral abnormalities were detected throughout the experimental period.

Table 5.1 - Ingredients and chemical composition (g/kg on dry matter basis) of the experimental diet

Composition	g/kg
Feed composition	
Tifton 85 hay	500
Ground corn	337
Soy bean meal	163
Chemical composition	
Dry matter	899
Organic matter (OM)	923
Crude protein (CP)	152
Ether extract (EE)	19
Neutral detergent fiber (NDF)	548
Acid detergent fiber (ADF)	256
Lignin (ADL)	42

5.2.2.3 Milk yield and composition

Milk production was measured twice weekly postpartum and for seven weeks. Milking was performed manually in individual ewe at 08:00 h. Before milking, teats were cleaned and disinfected with once-use cleaners that were put in warm water. Daily milk yield for each ewe was performed using weight suckling-weight method (WILLIAMS et al., 1979). Lambs were separated from their dams at 18:00 h at the previous day of milking and in the day after. The lambs were weighted at 08:00 h and were left to suckle their dams till satisfactions. They were weighed again and were kept in closed pens till next milking in the afternoon. In the mean time their dams were striped to estimate the stripping milk. The same procedure was followed again at 18:00 h in the same day. The daily milk yield was calculated by summing the weight of suckled milk (differences between lamb's weight before and after suckling) and the weight of striped milk in both morning and evening milking. Milk production was evaluated using a graduated cylinder (±5 mL). The amount of milk obtained

was adjusted for 24 h on weekly basis. Energy corrected milk (kg/d) was estimated as: = 0.3246*milk yield + (12.86 * fat yield) + (7.04*protein yield) (BERNARD, 1997). Milk energy value (kcal/kg) was calculated according to Baldi et al. (1992) as 203.8+ (8.36*fat %)+ (6.29*CP %). Milk samples (30 mL) from individual ewes, added with potassium dichromate and stored at 4 ◦C till analysis. Milk fat, protein, lactose, total solids, solid not-fat, somatic cell count (SCC) and pH were analyzed using the Milk-O-Scan (MilkoScan™ FT+, Hillerd, Denmark).

5.2.2.4. Body weight (BW) and body condition score (BCS)

Body weight was measured weekly before feeding using a digital standing scale. Body condition score (BCS) was recorded by three trained persons at the same time and at the commencement and the end of the study as well as at lambing. A 5-point scoring scale, with 1 being an emaciated or extremely thin ewe and 5 describing an obese or extremely fat ewe. The BCS of the ewes in this system was based on palpation of the tips of both the spinous and the transverse processes of the vertebrae, and the fullness of muscle and fat cover over and around the vertebrae in the loin region as described by Russel et al. (1969).

5.2.2.5. Lambs weight and performance

Lamb birth weight was recorded after 24 h of birth in order to allow sufficient chance for dams to recognize their lamb. Weaning weight (WW) was recorded when lambs reached eighth weeks of age. Average daily gain (ADG) was calculated using the weekly body weights for each lamb. For animal welfare reasons lambs were allowed to stay with dam from 18:00 h until 08:00 h every day except the days of estimating milk production. Lambs had no access to dam feeds. They had their own troughs and feeders with high concentrate meal (creep feeding) which started to be offered (adlibitum) when they reached 30 days old the creep feeding and chemical composition are summarized in Table 5.2. Water and mineral mixture were available ad lib for all lambs.

Table 5.2 - Per-parturient dietary ingredients (g/kg) and nutrient composition (DM based) of lambs creep feeding

Composition	g/kg
Creep composition	
Ground corn	700
Soybean meal	300
Chemical composition	
Dry matter (DM)	905
Organic matter (OM)	961
Crude protein (CP)	210
Ether extract (EE)	25
Neutral detergent fiber (NDF)	582
Acid detergent fiber (ADF)	75
Lignin (ADL)	7

5.2.2.6 Statistical analysis

Data were analyzed using PROC MIXED on SAS (2002).The model included effect of treatment, week, and their interaction, on different variables were tested in a repeated measure design as follows:

$$Y_{ijk} = \mu + T_i + W_j + (TW)_{ij} + A_{kt} + e_{ijk}$$

Where: μ is overall mean, T_i is a fixed effect of the i^{th} treatment (i=1 to 2), W_j is a fixed effect of the j^{th} week (j=1 to 8) , TW_{ij} is an interaction between i^{th} treatment and j^{th} week, A_{kt} is random effect of the animal (inside treatment) and e_{ijk} is random error assumed to be independent by and normally distributed with mean = 0 and variance = σ^2. The SCC was transformed by log10 (x + 10) and differences at the 5% level were considered significant.

5.3 Results

5.3.1 Propolis major components

The chemical composition and major components of Brazilian red propolis was analyzed by GC/MS and shown in Table 5.3. The relative percentage of the identified major compounds of the Brazilian propolis were isoflavonoid which presented almost 42.5% of total content of propolis especially edicarpin (13.52%), Vestitol (24.62%), formononetin (1.84%) and isoliquiritigenin (2.46%).

Table 5.3 - Identification of constituents of Brazilian red propolis (BRP) by GC/MS

Compounds	*t*R (min)*a*	(% area of each component)*e*
4,4' Bis[(Trimethylsilyl)Ethynyl]-2,2'-Bithiophene-5,5' Dicarbaldehyde	36.49	0.80
Silane, Trimethyl[5-Methyl-2-(1-Methylethyl)Phenoxy]*	37.41	4.97
Medicarpin	37.55	13.52
Benzenepropanoic Acid, 3,4-Bis[(Trimethylsilyl)Oxy]-, Trimethylsilyl Ester*	38.11	3.93
Benzenepropanoic Acid, 3,5-Bis[(Trimethylsilyl)Oxy]-, Trimethylsilyl Ester	38.24	16.49
Vestitol	38.37	24.62
4,4'-Bis[(Trimethylsilyl)Ethynyl]-2,2'-Bithiophene-5,5'-Dicarbaldehyde	39,07	7.07
Hydrocinnamic Acid, P-(Trimethylsiloxy)-, Trimethylsilyl Ester*	39.43	1.96
3,4-Dihydroxy-9-Methoxypterocarpan	39.68	1.51
3,8-Dihydroxy-9-Methoxypterocarpan (3-Hydroxy-8,9-Dimethoxypterocarpan)	40.13	0.79
9h-Fluorene-4,5-Diamine, N,N,N',N'-Tetramethyl*	40.51	2.88
Formononetin	40.86	1.84
Silane, 9h-Fluoren-9-Ylidenebis(Trimethyl)*	41.17	0.78
2,5,?-Tri-Oh-Phenylacetate 4tms	41.44	1.77
Isoliquiritigenin	41.74	2.64
2-Propenoic Acid, 3-(3,4,5-Trimethoxyphenyl)-, Methyl Ester*	42.02	0.70
Silane, Trimethyl[1-[(Trimethylsilyl)Ethynyl]-2-Naphthalenyl]*	44.91	3.30
Propanedioic Acid, Bis[(Trimethylsilyl)Oxy]-, Bis(Trimethylsilyl) Ester*	45.60	0.78
Beta-Amirin Trimethylsilyl Ether	47.57	2.33
Silane, (9,19-Cyclo-9.Beta.-Lanost-24-En-3.Beta.-Yloxy)Trimethyl*	48.11	3.09
9,19-Cyclolanostan-3-Ol, 24-Methylene-, (3.Beta.)*	48.78	1.21
Lup-20(29)-En-3-Yl Acetate	49.26	2.54
Solanesol	49.88	0.48

*t*R (min)*a* = retention time , min)
* Components were similarity more than 50% of the search results by comparison with data from literature and the profiles from the Nist 98 library.
*The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.

5.3.2 Effect of propolis on milk yield and composition

The effects of BRP on milk yield, milk composition and somatic cell counts are presented in Table 5.2 Propolis increased ($P < 0.05$) milk yield, and fat content in the treated ewes compared with control. Also, yield of fat, protein and lactose and ECM were increased ($P < 0.05$) by propolis treatment in comparison with control group. On the other hand, SCC was decreased ($P < 0.05$) by propolis supplementation while, there were no significant ($P > 0.05$) deference were observed for other milk constituents e.g. milk energy value and milk pH. Milk yields decreased ($P < 0.05$) towards as lactation advanced, the same trend was observed for protein yield and lactose yield. There was no significant ($P > 0.05$) effect of treatment x week's interaction for all tested variables.

Table 5.4 - milk yield, composition and somatic cell counts of Santa inês ewes supplemented with BRP

Variable	Treatment		SEM	*P*-value[a]		
	Control	Propolis		T	W	TxW
Milk yield, kg/d	1.12[b]	1.36[a]	0.68	0.03	0.02	0.63
Fat, %	3.21[b]	3.71[a]	0.17	0.04	0.07	0.98
Protein, %	5.71	5.60	0.09	0.46	0.15	0.91
Lactose, %	5.00	5.11	0.07	0.31	0.16	0.99
Total Solids,%	14.86	15.21	0.21	0.25	0.25	0.65
Solids not fat,%	11.58	11.58	0.08	0.99	0.47	0.89
Fat yield, g/d	35.05[b]	49.16[a]	3.04	0.02	0.53	0.81
Protein yield, g/d	62.67[b]	76.01[a]	3.99	0.03	0.04	0.77
Lactose yield, g/d	57.70[b]	69.38[a]	3.70	0.04	0.02	0.69
Energy corrected milk (kg/d)[1]	1.24[b]	1.61[a]	0.08	0.03	0.15	0.65
Milk energy value (kcal/kg)[2]	266.52	270.07	1.69	0.15	0.07	0.97
Log SCC	3.31[a]	2.97[b]	0.16	0. 05	0.25	0.22
pH	6.73	6.74	0.02	0.59	0.09	0.83

Energy corrected milk (kg/d)[1] = 0.3246*milk yield + (12.86 * fat yield) + (7.04*protein yield)
Milk energy value (kcal/kg)[2] = 203.8+(8.36*fat %)+(6.29*CP %).
Means with different letters within the row are different (P<0.05). SEM= stander error of mean, T= treatment, W= weeks , TxW= treatment weeks interaction

5.3.3 Effect of propolis on BW and BCS of ewes

Propolis did not affect significantly (P >0.05) on ewes BW while, it decreased significantly (P<0.05) after parturition and by advances of lactation Figure 5.2. Body condition score was improved (P<0.05) in propolis group compared with control, but no significant differences were observed by the advances of lactation.

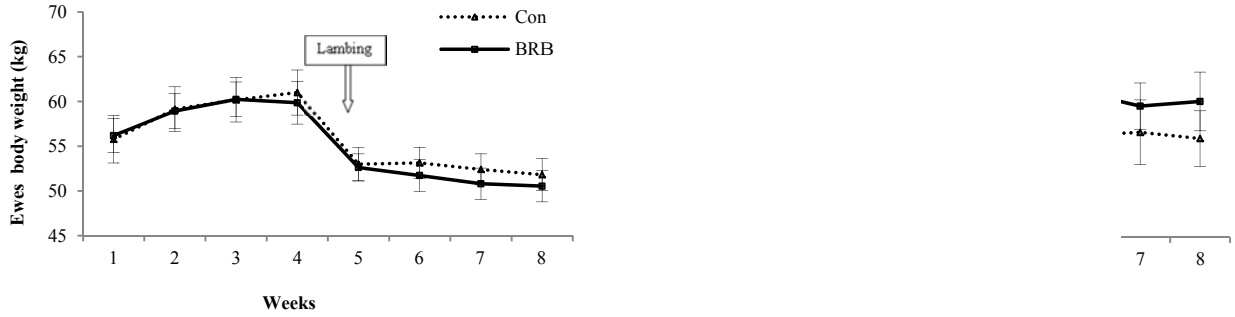

Figure 5.2 - Body weight and body condition score of Santa inês ewes supplemented with BRP before one month of parturition

5.3.4 Effect of propolis on lambs performance

Santa inês lambs performance was presented in Table 5.5 the results showed that, there were no significant differences (P>0.05) were observed for birth and weaning weight of lambs born from ewes supplemented with propolis while, ADG was increased ($P < 0.05$) to reach 0.20 vs 0.17kg/d in lambs born from ewes supplemented with propolis compared with control, while milk conversion ratio (MCR) milk consumed during 50 d: daily body weight gain, kg/kg was numerically higher (P<0.05) in control group than propolis group and it tended to decreased significantly by the advances of lactation weeks. No significant differences were observed by treatment x week's interaction.

Table 5.5 - Weaning weights and average daily gain (ADG) of Santa inês lambs of ewes supplemented with BRP

Variable	Treatment		SEM	*P*-value[a]		
	Control	Propolis		T	W	TxW
Birth weight (kg)	3.93	3.96	0.10	0.88	-	-
Weaning weight (kg)	13.99	14.50	0.65	0.70	-	-
Average daily gain(g/d)	170[b]	200[a]	0.80	0.04	0.13	0.65
Milk conversion ratio[1]	1.89[a]	1.58[b]	0.11	0.03	0.02	0.16

[1]Milk conversion ratio: milk consumed during 50 d: daily body weight gain, kg/kg., Animals started creep feeding after 30 days of therir age
Means with different letters within the row are different (P<0.05).
SEM= stander error of mean ,T= treatment, W= weeks, TxW= treatment weeks interaction

5.4 Discussion

5.4.1 Propolis chemical composition and major components.

Brazilian red propolis has been widely studied to elucidate its chemical composition and 12 types of Brazilian propolis have been characterized and classified into types 1–12 (PARK et al., 2000). Brazilian propolis is quite diverse in chemical composition, due to Brazil's rich biodiversity, which needs to be investigated as a source of new bioactive substances. The results of GC/MS analysis of the tested Brazilian red propolis confirmed that this type was not included among these 12 types that classified by Park et al. (2000). The current propolis was the type 13 (SILVA et al., 2007), which first classified by Alencar et al. (2007) since 42.5% of the total content of propolis were isoflavonoides (Medicarpin 13.52%, Vestitol 24.6%, Formononetin 1.84% and Isoliquiritigenin 2.64% respectively) as shown in Table 5.2. The Vestitol was the most abundant compounds which confirmed by the GC–MS analysis that never before reported in the previously 12 Brazilian propolis types (PARK et al., 2002; ALENCAR et al., 2007). The data suggest that these major compounds may be associated with the biological properties and main activity of these varieties of propolis. There are several studies indicated that isoflavones have antimicrobial, antifungal, anticancer, antiprotozoal, osteoporosis, antioxidant action (ALENCAR et al., 2007) and relieve the symptoms of menopause. Thus, consumption of foods containing isoflavone phytoestrogens has been associated with a variety of health benefits (CATTANI et al., 2012; ALENCAR et al., 2007; KANO et al., 2006). isoflavones has been indentified in milk and the content of isoflavonoides in milk can vary depending on the composition of the diet that cows are fed, and organic milk in general contains higher concentrations of isoflavones which have benefit impact even in animal and human health (HOIKKALA et al., 2007; ANTIGNAC et al., 2004).

5.4.2. Propolis affects on milk yield composition and somatic cell counts

Milk yield was increased ($P<0.05$) by propolis administration, while decreased ($P < 0.05$) by the advances of lactation (Table 5.4). This results are agreement with Freitas et al. (2009) who concluded that the addition of the ethanolic extract of propolis had significant effect ($P < 0.05$) on milk production (25.62 vs. 22.62 kg/day) in Holstein dairy cows. This increment of milk production could be attributed to assumption, which proposed that propolis

is an alternative to the use of dietary antibiotics (ÍTAVO et al., 2011). According to Mirzoeva et al. (1997), propolis has bacteriostatic activity against gram-positive and some gram-negative bacteria as the action mechanism of ionophores. Science propolis can inhibit the growth of gram positive bacteria, which produce more ammonia, methane and lactate than gram negative species, which increased feed efficiency (RUSSELL; STROBEL, 1989). Thus, the reduction of the number of these bacteria (methane producing) reduces the energy loss up to 13% related to feed intake which interne providing larger amount of energy for production as milk (VAN SOEST, 1994).

The reduction of amino acid deamination process may also, associated with an increase in milk production, since this process entails to economy the animal energy. According to Simas and Nussio (2001), a reduction of ammonia production up to 50% by ionophores, occurs by decreasing the number of gram-positive bacteria and increased microbial protein. The action of propolis on reducing deamination was observed in studies conducted by Oliveira et al. (2007), who demonstrated that propolis had the highest deamination effect when compared with monensin *in vitro*. Similarly, Stradiotti Junior et al. (2004a) reported that ethanolic extract of propolis (EEP) 30% reduced deamination of amino acids in the rumen of cattle and enhanced ruminal protein escape, with consequent improvement of production efficiency of ruminants.

On the other hand, Stelzer et al. (2009) in his experiment which conducted using two levels of concentrate (20 and 40% dry matter) and presence or absence of EEP in the diet at 30% w/v for Holstein dairy cows observed that, the addition of propolis does not alter the dry matter intake, digestibility and performance of cows producing above 20 kg of milk/day. The same finding was observed by Lana et al. (2005), who demonstrated that, Alpine dairy goats which supplemented with soybean oil (0 or 120 g/animal/day) and/or EEP (0 or 10 mL/animal/day). Soybean oil was more effective to increase milk yield than the EEP. On the other hand, Stelzer et al. (2009) concluded that 30% of EEP did not affect on milk production, production of milk corrected to 3.5% of Holstein dairy cows. The current results indicated that milk yield was affected significantly with stage of lactation Figure 5.2. Where it reached its peak at three weeks after lambing, persisted for another two weeks, and then declined till the end of lactation, which in agreement with Sevi et al. (2004).

Although propolis mode of action is similar to ionophores (ÍTAVO et al., 2011) which accompanied with decreasing milk fat percentage (DUBUC et al., 2009) but such as effect did not observed in our study where, fat content was increased ($P < 0.05$) by propolis inclusion.this finding is consistent with Andrighetto et al. (2005) who working with dairy

buffaloes and observed positive increase of monensin on the milk fat. The reduction in the proportion acetate: propionate in the rumen by the action of ionophores does not appear to be associated with milk fat (FREITAS et al., 2009), since that is directly related to the increase in the proportion of propionate and with no reduction in the proportion of acetate (VAN SOEST, 1994). Such increase of milk fat content in propolis group could be attributed to the increasing of total short chain fatty acids by EEP as mentioned by Stradiotti Junior et al. (2004a). On the other hand (FREITAS et al., 2009; STELZER et al., 2009) observed that, propolis did not affect on milk fat content. Milk fat yield of propolis group was increased ($P < 0.05$), this increase is a reflect of positive effect of propolis treatment on both milk yield and fat content science the calculation of milk fat yield is dependent on milk yield and fat content.

Both protein and lactose yield were increased ($P < 0.05$) by propolis treatment and decrease ($P < 0.05$) by the advances of lactation weeks.this increase of protein and lactose yields could be related to the significant increase of milk yield in the propolis group. Also Freitas et al. (2009) mentioned that, the increase of protein yield possibly occurred due to amino acid additions to mammary gland, promoted by low fermentation rate of protein dietary and reduction in gas production, enabling greater protein escape to the small intestine, where it will be digested and its constituents absorbed by the intestinal epithelium. The same results were obtained by Stradiotti Junior (2004a). On the other hand, Freitas et al. (2009); Stelzer et al. (2009) found that EEP did not affect on the content of lactose. Regarding the effect of weeks of lactation on protein and lactose yields the present result indicate that both protein and lactose yield tended to decrease by the advances of lactation which agrees with results of Sevi et al. (2004). This effect may be related to the significant decrease of milk yield by lactation progressed. Since milk yield and fat content increased by propolis treatment as shown above which in turn led to increase energy corrected milk (ECM) as presented in Table 5. 2. Because ECM is a correction of milk yield for its content in fat and protein .Data of the current study were consistent with those of Casals et al. (1999), who used different formula to calculate ECM and found that it increased over the whole lactation period.

Moreover, propolis treatment resulted in decreasing ($P < 0.05$) SCC. This result may suggest the poetical of propolis against mastitis infection since the coloration between SCC and mastitis has been well documented (GREEN et al., 2004)**.** The antimicrobial activity of propolis ethanolic extract against *Candida albicans*, *Escherichia coli*, *Staphylococcus sp* and *Streptococcus sp,* the major microorganisms that causing mastitis was observed *in vitro* as well as *in vivo* even in ovine or bovine species (MERESTA et al., 1989; PINTO et al., 2001;

LOGUERCIO et al., 2006 ; SILVA et al., 2012). On the other hand, Vargas et al. (2002); Freitas et al. (2009) concluded that propolis did not affect on SCC.

5.4.3. Effect of propolis on BW and BCS of dams

Even propolis did not affect on BW, but it affect ($P < 0.05$) on BCS of the ewes, and both parameters affected ($P < 0.05$) by parturition (Figure 5.2). These results could suggest that, propolis able to improve the quantity of ewes' body reserve (mainly fat, as the largest source of energy, but also the major reserve of protein in the body and the skeletal muscle) which intern affect positively on milk yield and fat content compared with control group. Since BCS methodology estimate the quantity of body energy reserves which has a definite effect on the reproductive and production efficiency of animals (CALDEIRA et al., 2007). Such effect of propolis could be due to several bioactivities of the propolis extract. For example, the propolis extract has anti-microbial, anti-inflammatory, anti-oxidant, and anti-viral properties (ALENCAR et al., 2007). In ruminants propolis has been used in the modification of rumen fermentation (OZTURK et al., 2010). Pires do Prado et al. (2010) reported that increases in total and intestinal digestibility, higher concentration of digestible energy and apparent increase in the flow of intestinal protein based diets with forage for buffalo fed with two concentrations of propolis 0,018 mg/g of LLOSC1 and 0,011 mg/g of LLOSB3.

After parturition, it is well known that the metabolic requirements increase in relation to the lactation process and the loss weight which occurs regarding placenta and fetal fluid that may induce a decrease in the body weight and BCS in Figure 5.2. In the current study, even both BW and BCS were decreased but BCS of propolis treated group still highly ($P < 0.05$) than control which prove that propolis may improve the quantity of ewes body reserve and enhance perseverance of energy body loss even after parturition.

5.4.4. Effect of propolis on lambs performance

Average daily gain of lambs born from ewes treated with propolis was higher ($P<0.05$) than control, Table 5. 5. This effect of propolis could be attributed to the increasing milk production and the improvement of milk quality regarding propolis treatment. Other

explanation may be due to propolis bioactive components such as isoflavonoids, which can be transferred into the milk and the concentration can vary depending on the composition of the diet that cows are fed (SKAANILD; NIELSEN, 2010).Where isoflavonoids have similar structure as estrogenic hormones (SONNENBICHLER; POHL, 1980). As such, isoflavonoids have been postulated to possess anabolic effects as estrogenic compounds (HAVSTEEN, 2002). Our results were in agreement with Zawadzki et al.(2011) who demonstrated that propolis extract in the diets of feedlot finished bulls increased ($P < 0.05$) ADG. Inconsistent to these results, Ítavo et al. (2011) and Sarker and Yang (2010) reported that propolis did not affect on ADG even in lambs or calves respectively.

The significant ($P < 0.05$) increase of MCR of lambs, probably due to higher energy corrected milk as mentioned in Table 5.5 or may be related to the increasing ewes milk yield and lambs ADG since MCR is a ratio between of milk consumed (suckled) to lamb daily gain. These results are in agreement with Zawadzki et al. (2011) who concluded that the addition of propolis extract to Nellore bulls diet improved ($P < 0.01$) feed conversion ratio. Regarding to the effect of weeks of lactation on milk conversion ratio, the results suggested that MCR decreased ($P < 0.05$) by the advances of lactation and this could be attributed to the gradual decrease of milk yield.

5.6 Conclusions

The oral administration of Brazilian red propolis increased milk yield and improved the milk quality which in turn led to enhance the lamb's performance.

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6 GENERAL CONCLUSIONS

- The major compounds of the Brazilian red propolis were isoflavonoids while the fatty acids were the major in the Egyptian brown propolis. Such compounds are responsible for the biological activity of each type of propolis.
- Propolis reduced CH_4 emission and enhanced the rumen TDOM
- Propolis was capable of improving the rumen fermentation, increasing the individual and total SCFA production and promising CH_4 mitigating agent in ruminants.
- Oral administration of such natural product (BRP) does not affect negatively reproductive performance of Santa inês ewes.
- Brazilain red propolis might be act as an anti-stress agent.
- Propolis had a good impact on ewe's health and it can be used as promising feed additive during a critical period such as flushing period.
- propolis presented efficacy to control sheep helminthiasis
- The oral administration of Brazilian red propolis increased milk yield and improved the milk quality, it might be act as an anti mastites which in turn led to enhance the lamb's performance.