UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

QUANTIFICAÇÃO E CARACTERIZAÇÃO DOS COMPOSTOS ATIVOS DA PRÓPOLIS E SEUS EFEITOS SOBRE A NUTRIÇÃO E QUALIDADE DO LEITE DE VACAS E CEPAS BACTERIANAS DO RÚMEN

Autora: Sílvia Cristina de Aguiar
Orientadora: Prof.ª Dr.ª Lúcia Maria Zeoula

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Tese apresentada, como parte das exigências para obtenção do título de DOUTORA EM ZOOTECNIA, no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá – Área de concentração Produção Animal.

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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

APROVADA em 07 de agosto de 2012.

Prof. Dr. Antonio Ferriani Branco

Profª Drª Mara Lane Carvalho Cardoso

Drª Evelyne Damiano Forano

Dr. Pedro Braga Arcuri

Profª Drª Lúcia Maria Zeoula
(Orientadora)

"O que for a profundeza do teu ser, assim será teu desejo.
O que for o teu desejo, assim será tua vontade.
O que for a tua vontade, assim serão teus atos.
O que forem teus atos, assim será teu destino"

- Brihadarayaka Upanishad IV, 4.5

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BIOGRAFIA

Sílvia Cristina de Aguiar, filha de Antonio Carlos de Aguiar e Mara Gisela de Aguiar, nasceu em Guaíra - Paraná, no dia 14 de abril de 1981.

Em dezembro de 2005, concluiu o Curso de Zootecnia, pela Universidade Estadual de Maringá.

Em março de 2007, iniciou no Programa de Pós-Graduação em Zootecnia, em nível de Mestrado, área de Concentração Produção Animal, na Universidade Estadual de Maringá, realizando estudos na área de Nutrição de Ruminantes.

No mês de abril de 2009, submeteu-se à banca examinadora para defesa da Dissertação de mestrado.

Em março de 2009, iniciou no Programa de Pós-graduação em Zootecnia, em nível de Doutorado, área de concentração Produção Animal, na Universidade Estadual de Maringá, realizando estudos na área de Nutrição de Ruminantes.

No mês de junho de 2012, submeteu-se à banca examinadora para o Exame Geral de Qualificação, como parte das exigências para a obtenção do título de Doutor em Zootecnia.

No mês de agosto de 2012, submeteu-se à banca para defesa da Tese.

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RESUMO

Foram realizados cinco experimentos, com o objetivo de quantificar e caracterizar os compostos fenólicos presentes em diferentes produtos à base de própolis (LLOS) e estudar seus efeitos sobre a digestibilidade, parâmetros ruminais e sanguíneos, eficiência de síntese de proteína microbiana na alimentação de vacas leiteiras, assim como avaliar seus efeitos sobre a composição em ácidos graxos (AG) e capacidade antioxidante do leite produzido por estas vacas. Também foi verificada a atividade antimicrobiana destes extratos de própolis sobre cepas bacterianas ruminais. O primeiro experimento teve como objetivo desenvolver e validar um método para a separação e quantificação dos compostos fenólicos de doze extratos à base de própolis obtidos com diferentes condições de extração, através de CLAE. Para a validação, foram analisadas a linearidade, a precisão (repetibilidade, precisão intermediária e reprodutibilidade), o limite de detecção e de quantificação, a recuperação/exatidão e a estabilidade do analito. A técnica para quantificação por meio de CLAE foi desenvolvida e validada propiciando segurança quanto à quantificação dos flavonoides e ácidos fenólicos totais presentes nos diversos extratos de própolis. As diferentes concentrações de própolis e teores alcoólicos interferiram na extração dos compostos ativos da própolis, podendo alterar a sua composição química e atividades biológicas. No segundo experimento, objetivou-se obter uma caracterização fenólica dos extratos de própolis através de CLAE . Em relação aos ácidos fenólicos, quatro compostos foram identificados nos doze extratos (ácido cafeico, ácido *p*-cumárico, CAPE e Artepillin C), enquanto alguns compostos foram detectados apenas em alguns extratos (ácido gálico, ácido clorogênico). Dentre os flavonoides, apigenina, pinocembrina e crisina foram detectados em todos os extratos e a galangina e acacetina foram detectadas na maioria dos extratos; no entanto, a naringenina foi identificada apenas em dois extratos. As concentrações de própolis e teor alcoólico influenciaram diretamente na extração de

alguns compostos fenólicos, alterando a composição química dos extratos de própolis. No terceiro experimento, três produtos à base de própolis foram adicionados às dietas para vacas em lactação, com o intuito de estimar o consumo, digestibilidade total (DT), ruminal (DR) e intestinal (DI), pH e produção de nitrogênio amoniacal ($N\text{-}NH_3$) ruminal, eficiência de síntese microbiana e parâmetros sanguíneos. Foram utilizadas quatro vacas da raça Holandesa com PC médio de 550 kg ± 34,16 kg, canuladas no rúmen, em um quadrado latino 4x4 (quatro tratamentos e quatro períodos). A dieta continha 59,19% de silagem de milho e 40,81% de concentrado, diferindo com a adição ou não dos LLOS: controle (sem aditivo), LLOS B1, LLOS C1 e LLOS C3. O pH ruminal, a eficiência de síntese microbiana e os parâmetros sanguíneos não foram influenciados ($P>0,05$) pela adição dos LLOS, mas houve efeito dos produtos à base de própolis ($P<0,05$) sobre o consumo, DT, DR, DI e para a produção de $N\text{-}NH_3$. Os LLOS proporcionaram menor ($P<0,05$) DR da PB, sendo que o LLOS C1 reduziu a produção de $N\text{-}NH_3$ ruminal e o LLOS B1 aumentou a DI da PB em relação à dieta controle. A adição dos produtos LLOS interferem no metabolismo ruminal e o produto LLOS C1 teve efeito positivo no metabolismo do nitrogênio, reduzindo as perdas de N na forma de NH_3 no rúmen. No quarto experimento, avaliaram-se os efeitos da adição dos LLOS B1, C1 e C3 sobre a qualidade, composição em ácidos graxos (AG) e atividade antioxidante nas amostras de leite das vacas utilizadas anteriormente. Os LLOS não influenciaram ($P>0,05$) a qualidade e a contagem de células somáticas das amostras de leite. Observou-se inversão nos teores de gordura e proteína do leite em todos os tratamentos, que provavelmente ocorreu pela adição de óleo de soja à dieta. A adição de própolis alterou ($P<0,05$) a composição de AG e aumentou a capacidade antioxidante do leite. Na menor concentração de flavonoides do produto LLOS, verificou-se aumento ($P<0,05$) no total de AG poli-insaturados e AG monoinsaturados, além de reduzir o total de AG saturados. Na maior concentração de flavonoides, registrou-se maior teor do isômero *cis*-9,*trans*-11 (CLA) em relação aos demais tratamentos, seguido pela concentração intermediária de flavonoides nos produtos. A adição dos LLOS à dieta reduziu a razão n-6/n-3 quando comparada ao controle. Para todos os LLOS, verificou-se aumento ($P<0,05$) na capacidade antioxidante do leite em relação à dieta controle. No quinto experimento, avaliou-se a atividade antimicrobiana dos extratos de própolis sobre cepas bacterianas do rúmen. Os extratos inibiram o crescimento de *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *R. albus* 7, *Butyrivibrio fibrisolvens*,

Prevotella albensis, *P. ruminicola*, *Peptostreptococcus* sp., *Clostridium aminophilum* and *Streptococcus bovis*, enquanto *R. albus* 20, *P. bryantii* e *Ruminobacter amylophilus* foram resistentes a todos os extratos. O potencial de inibição foi influenciado pelas condições de extração dos compostos fenólicos presentes na própolis. Conclui-se que os produtos LLOS podem trazer benefícios quando fornecido a vacas leiteiras, uma vez que melhorou o desempenho animal e a qualidade do leite de vacas, entretanto, mais estudos devem ser realizados para melhor entendimento de sua atividade antimicrobiana.

Palavras-chave: aditivo, atividade bacteriana, CLA, extratos de própolis, flavonoides, oxidação lipídica

ABSTRACT

Five experiments were carried out in order to quantify the phenolic compounds present in different propolis-based products (LLOS) and study its effects on digestibility, ruminal and blood parameters and efficiency microbial protein synthesis in dairy cows, and assess their effects on fatty acids composition (FA) and antioxidant capacity in milk produced by these cows. Also, the antimicrobial activity of propolis extracts on ruminal bacterial strains was evaluated.The first experiment aimed to develop and validate a method for separation and quantification of phenolic compounds in twelve propolis extracts obtained by different extraction conditions using HPLC. For validation, we analyzed the linearity, precision (repeatability, intermediate precision and reproducibility), limit of detection and quantification, recovery/accuracy and stability of the analyte. The developed method was validated providing certainty in the quantification of total flavonoids and phenolic acids present in different propolis extracts. The different concentrations of propolis and alcohol levels interfere in the extraction of the active compounds of propolis, which may change its chemical composition and biological properties. In the second experiment, the objective was to obtain a phenolic fingerprint of propolis extracts using HPLC. Regarding the phenolic acids, four compounds were identified in the twelve extracts (caffeic acid, *p*-coumaric acid, CAPE and Artepillin C), while some compounds were detected only in some extracts (gallic acid, chlorogenic acid). For flavonoids, apigenin, pinocembrin, and chrysin were detected in all extracts and galangin and acacetin were detected in most extracts; however naringenin has been identified only in two extracts. The different concentrations of propolis and alcohol levels directly influence the extraction of some phenolic compounds, which alters the chemical composition of the propolis extracts. In the third experiment, propolis-based products (LLOS) were evaluated, in order to estimate the feed intake, total, (TD), ruminal (RD) and intestinal (ID) digestibility, pH

and ruminal ammonia-nitrogen production (NH_3-N), rumen microbial synthesis and blood parameters. Four Holstein cows, weighing 550 ± 34.16 kg of BW and cannulated in the rumen were used, and distributed in a 4 x 4 Latin Square. The diet contained 59.19% of corn silage and 40.81% of concentrate, differing with the addition or not of LLOS: control (no LLOS), LLOS B1, LLOS C1 and LLOS C3. The ruminal pH, efficiency of microbial protein synthesis and blood parameters were not affected (P> 0.05) by the addition of LLOS, but there was an effect of the propolis-based products (P <0.05) on feed intake, TD, RD, ID and NH_3-N production. The LLOS provided lower (P <0.05) RD of CP, the LLOS C1 reduced the ruminal NH_3-N production and the LLOS B1 increased the ID of the CP compared to the control diet. The addition of the LLOS products interfere with ruminal metabolism and the product LLOS C1 had a positive effect on nitrogen metabolism, because it reduced the losses of N in the NH_3 form, in the rumen. In the fourth experiment, the effects of the LLOS were evaluated on the quality, fatty acid (FA) composition and antioxidant capacity in milk samples from cows in the previous experiment. The LLOS did not affect (P> 0.05) the quality and somatic cell count of milk samples. It was observed an inversion in the milk fat and protein contents in all treatments, which was probably due to the addition of soybean oil to diet. The addition of propolis affected (P <0.05) the FA composition and increased the antioxidant capacity of milk. At the lowest concentration of flavonoids there was an increase (P <0.05) in total polyunsaturated and monounsaturated FA, with reduction in the total saturated FA. At the highest concentration of flavonoids, there was a higher content of the *cis-9,trans-11* isomer (CLA) compared to the other treatments, followed by the intermediate concentration of flavonoids in the products. The addition of LLOS to diet reduced the n-6/n-3 ratio when compared to control diet. For all LLOS, there was an increase (P <0.05) in the antioxidant capacity of milk in relation to control diet. In the fifth experiment, the antimicrobial activity the propolis extracts was evaluated on rumen bacterial strains. The different propolis extracts inhibited the growth of *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *R. albus* 7, *Butyrivibrio fibrisolvens*, *Prevotella albensis*, *P. ruminicola*, *Peptostreptococcus* sp., *Clostridium aminophilum* and *Streptococcus bovis*, while *R. albus* 20, *P. bryantii* and *Ruminobacter amylophilus* were resistant to all the extracts. The potential of inhibition was influenced by the extraction conditions of the phenolic compounds present in propolis. It can be concluded that the LLOS can benefit when given to dairy cows, since improve animal

performance and the milk quality from dairy cows; however, more studies are needed to better understanding of its antimicrobial activity.

Keywords: additive, antibacterial activity, CLA, flavonoids, lipid oxidation, propolis extracts

CAPÍTULO I

INTRODUÇÃO

Própolis e seus compostos fenólicos

A palavra "própolis" deriva do grego *pro* ("a frente de", "em defesa de") e *polis* ("comunidade" ou "cidade") e consiste, como o próprio nome diz, em uma substância para a defesa da colmeia (Castaldo & Capasso, 2002). A própolis é um material resinoso coletado pelas abelhas operárias de brotos e secreções de árvores de inúmeras espécies. Com o objetivo de produzir a própolis, as abelhas também podem coletar materiais secretados ativamente pelas plantas ou de seus exsudatos (material lipofílico nas folhas, resinas, etc.) (Bankova et al., 2000). Uma vez coletado, este material é enriquecido com secreções salivares e enzimáticas e é utilizado pelas abelhas para vedar a parede da colmeia, preencher lacunas ou fendas e embalsamar insetos invasores mortos dentro da colmeia (Castaldo & Capasso, 2002).

A resina contida na própolis é coletada na vegetação das cercanias da colmeia. O espectro de voo de uma abelha *Apis mellifera* abrange um raio de cerca de 4-5 km em torno da colmeia, onde abelhas campeiras coletam pólen e néctar para alimentação, bem como resina para a própolis (Menezes, 2005). Não são conhecidos os fatores que direcionam a preferência das abelhas coletoras de resina por uma determinada fonte vegetal, mas sabe-se que elas são seletivas nesta coleta (Salatino et al., 2005; Teixeira et al., 2005). Possivelmente, esta escolha esteja relacionada com a atividade antimicrobiana da resina, uma vez que as abelhas utilizam a própolis como um antisséptico (Sahinler & Kaftanoglu, 2005), conforme discutido acima.

A própolis não é somente um material de construção e sim, a mais importante "arma biológica" das abelhas contra microrganismos patogênicos e tem sido utilizada na medicina humana desde a antiguidade (Bankova, 2005a). Os egípcios conheciam muito

bem as propriedades antiputrefativas da própolis e a utilizavam para embalsamar cadáveres. A própolis foi reconhecida por suas propriedades medicinais através dos físicos gregos e romanos Aristóteles, Dioscorides, Plínio e Galeno e foi aplicada como antisséptico e cicatrizante no tratamento de feridas e como desinfetante bucal, sendo essas aplicações perpetuadas na Idade Média e entre físicos árabes. A própolis também foi reconhecida por outros povos não relacionados às civilizações do Velho Mundo: os Incas utilizavam a própolis como um agente antipirético, e as farmacopeias londrinas do século XVII listaram a própolis como uma droga oficial. Entre os séculos XVII e XX, a própolis se tornou muito popular na Europa por conta da sua atividade antibacteriana (Castaldo & Capasso, 2002). No Brasil, o interesse pela própolis aconteceu somente na década de 1980 com o trabalho pioneiro de Ernesto Ulrich Breyer, demonstrando em seu livro, "Abelhas e saúde", as propriedades terapêuticas da própolis e sua utilização como antibiótico natural (Lustosa et al., 2008).

Os principais compostos químicos isolados da própolis até o momento podem ser organizados em alguns grupos principais como: ácidos e ésteres alifáticos, ácidos e ésteres aromáticos, açúcares, alcoóis, aldeídos, ácidos graxos, aminoácidos, esteroides, cetonas, flavonoides (flavonas, flavonóis e flavononas), terpenoides, proteínas, vitaminas B1, B2, B6, C, E, bem como diversos minerais, como magnésio, cálcio, ferro e zinco. Embora a composição química de própolis foi esclarecida em certa medida nos últimos anos, ainda existe um problema: a variabilidade marcante da sua composição química, dependendo do local de colheita (Menezes, 2005; Castaldo & Capasso, 2002).

A composição química da própolis depende da especificidade da flora no local de coleta e, portanto, das características geográficas e climáticas deste local, sendo este o maior problema na padronização da própolis (Bankova, 2005b). Segundo Bankova (2005a), é importante que os pesquisadores que estudam a atividade biológica da própolis estejam cientes sobre a existência do problema de padronização e que sejam capazes de distinguir entre os diferentes tipos de própolis. É essencial ter dados detalhados e comparativos que sejam confiáveis sobre cada tipo de atividade biológica, combinado com dados químicos, a fim de decidir se algumas áreas específicas de aplicação de um tipo particular de própolis possam ser formuladas como preferíveis. Os testes biológicos devem ser realizados com própolis quimicamente caracterizada e, se possível, quimicamente padronizada, uma vez que os compostos ativos da própolis são dependentes desde a sua produção até a sua extração.

Critérios confiáveis para a padronização química dos diferentes tipos de própolis são necessários. A própolis não pode ser utilizada em sua forma bruta e deve ser purificada por extração com solventes. Este processo deve remover o material inerte e preservar as frações polifenólicas. Uma extração etanólica em várias etapas é particularmente adequada para se obter extratos de própolis "desparafinados" e ricos em componentes polifenólicos (Pietta et al., 2002). Entretanto, dependendo do tipo e da quantidade de solvente empregados assim como da quantidade de própolis utilizada para a extração, os extratos podem apresentar composições químicas e atividades biológicas distintas (Park & Ikegaki, 1998; Kumazawa et al., 2004; Trusheva et al, 2007; Cottica et al., 2011; Sforcin & Bankova, 2011), o que dificulta ainda mais a padronização da própolis.

A própolis tem sido objeto de estudos farmacológicos devido às suas propriedades antimicrobiana, antifúngica, antiviral, anti-inflamatória, hepatoprotetora, antioxidante, antitumoral, imunomodulatória, entre outras (Kumazawa et al., 2004; Bankova, 2005a; Kosalec et al., 2005; Alencar et al., 2005). Esse potencial biológico se deve a um sinergismo que ocorre entre seus muitos constituintes (Marcucci, 1996), dentre eles, os compostos fenólicos.

Dentre os principais compostos químicos da própolis, certamente os que vêm chamando mais atenção dos pesquisadores são os flavonoides (Lustosa et al., 2008). A eles, bem como aos ácidos fenólicos, são atribuídas às propriedades antimicrobiana, antiviral e antioxidante (Volpi & Bergonzini, 2006).

Pelo menos 200 compostos foram identificados em diferentes amostras de própolis, com mais de 100 em cada uma (Marcucci et al., 2001). Em 2000, doze tipos distintos de própolis brasileira foram quimicamente caracterizados e classificados de tipo 1 a 12 (Alencar et al., 2005; Hayacibara et al., 2005). As amostras tropicais de própolis, especialmente as brasileiras, têm mostrado diferenças significativas nas suas composições químicas em relação à própolis da zona temperada. Por essa razão, a própolis brasileira tornou-se objeto de grande interesse por parte dos cientistas (Trusheva et al., 2006). A própolis verde brasileira produzida em São Paulo e Minas Gerais, por exemplo, é constituída principalmente de derivados prenilados do ácido *p*-cumárico e possui grande quantidade de flavonoides, muitos dos quais não estão presentes em própolis da Europa, América do Norte e Ásia (Simões et al., 2004). Dentre os flavonoides identificados na própolis brasileira destacam a acacetina, pinobanksina,

kampferol, apigenina, pinocembrina, crisina, galangina, isossacuranetina e betuletol (Park et al., 2002a; Park et al., 2002b; Sawaya et al., 2004; Funari & Ferro, 2006; Chang et al., 2008; Tavares et al., 2010; Cottica et al., 2011) (Figura 1).

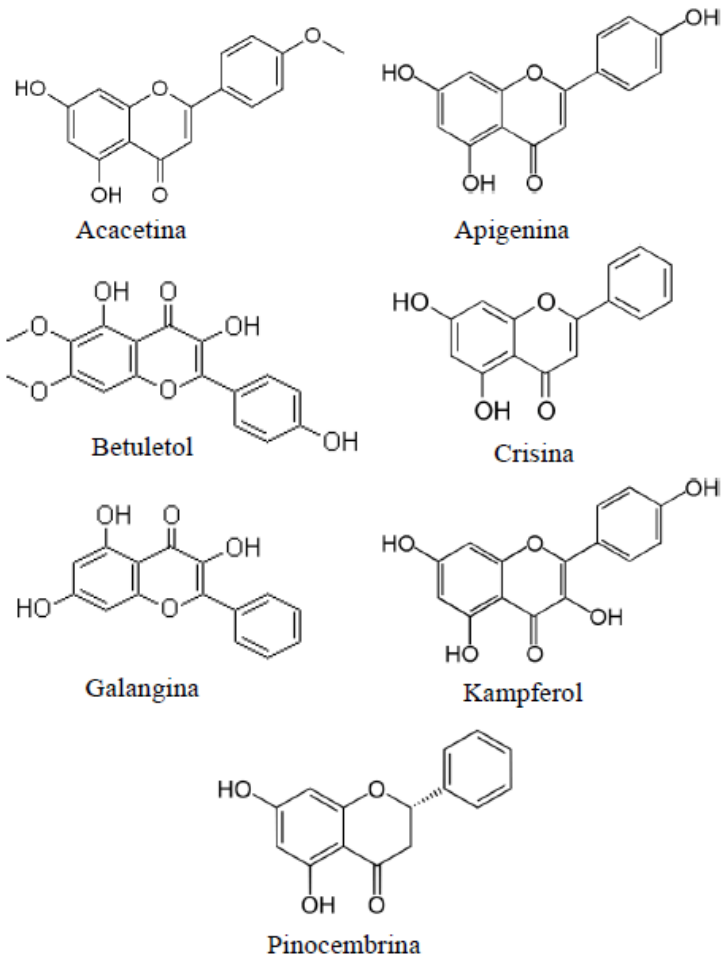

Figura 1 – Estrutura química de alguns flavonoides encontrados na própolis brasileira.

A ingestão de flavonoides interfere em diversos processos fisiológicos, auxiliando na absorção e na ação de vitaminas, atuando nos processos de cicatrização como antioxidantes, além de apresentarem atividade antimicrobiana e moduladora do sistema imune (Williams et al., 2004). Apesar de os flavonoides serem os componentes da própolis mais extensivamente estudados, eles não são os únicos responsáveis pelas suas propriedades farmacológicas. Diversos compostos são relacionados com as propriedades medicinais da própolis (Awale et al., 2005) como, por exemplo, os ácidos fenólicos.

Os principais ácidos fenólicos encontrados na própolis brasileira são: ácido cinâmico e derivados (ácido cafeico, ácido *p*-cumárico, drupanina, bacarina, entre outros), ácido benzoico, ácido 3-prenil-hidroxicinâmico, ácido clorogênico, ácido ferúlico, ácido trans-cinâmico, éster fenetil do ácido cafeico (CAPE) e Artepillin C (ácido 3,5-diprenil-4-hidroxicinâmico) (Park et al., 2002a, b; Funari & Ferro, 2006; Sousa et al. 2007; Chang et al., 2008; Salomão et al., 2008; Tavares et al., 2010; Chikaraishi et al., 2010).

Dentre os compostos fenólicos encontrados na própolis brasileira, o Artepillin C (Figura 2) é o que mais atrai a atenção de pesquisadores da área, sendo bastante estudado nos últimos anos. O Artepillin C foi encontrado pela primeira vez na própolis brasileira em 1994, por Aga e colaboradores (Aga et al., 1994), os quais descobriram sua atividade antibacteriana. Esta descoberta, juntamente com alguma semelhança estrutural com o CAPE (a presença do sistema trans-cinamoil), levou ao aumento do interesse por este composto e em pouco tempo diversas atividades farmacológicas importantes foram encontradas e alguns estudos revelando os mecanismos de ação do Artepillin C foram realizados (Bankova, 2009). Recentemente, várias atividades biológicas têm sido atribuídas ao Artepillin C, tais como atividades antioxidante, antimicrobiana e antitumoral (Estrada et al., 2008).

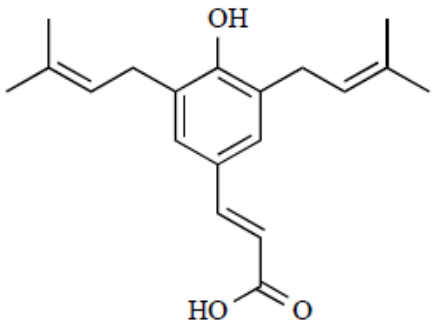

Figura 2 – Estrutura química do Artepillin C

Existem muitos trabalhos na literatura que relatam a presença do Artepillin C na própolis brasileira. Chang et al. (2008) analisaram extratos em etanol de própolis verde brasileira (*Baccharis dracunculifolia*) e identificaram o Artepillin C (63% do pico-base) nos extratos analisados. Outros pesquisadores também encontraram tal composto em extratos de própolis provenientes do Brasil, sendo que o Artepillin C se tornou um

importante fator como indicador de qualidade da própolis brasileira (Funari & Ferro, 2006; Sousa et al., 2007; Salomão et al., 2008; Estrada et al., 2008; Tavares et al., 2010; Chikaraishi et al., 2010).

De acordo com Bankova (2005a) a padronização química universal de um produto tão variável como a própolis é impossível, mas existe sim uma possível padronização, através da formulação de diferentes tipos de própolis de acordo com sua origem botânica e composição química correspondente. Portanto, fica claro que a determinação de sua composição é de extrema importância para melhor entendimento de suas propriedades biológicas, assim como seus efeitos. Sabe-se que os compostos fenólicos são os principais compostos ativos da própolis, portanto, sua identificação e quantificação são fatores-chave para a elucidação de muitos questionamentos envolvendo suas propriedades biológicas e mecanismos de ação, assim como para melhor aproveitamento dos benefícios que a própolis pode oferecer.

Própolis na nutrição de ruminantes

Nos últimos anos, observou-se maior preocupação com o uso de antibióticos na agricultura e agropecuária, em virtude da resistência adquirida pelos microrganismos a estes agentes antimicrobianos. Esta preocupação foi reforçada pela observação de que a avoparcina, um antibiótico utilizado na Europa para promover o crescimento animal, conduziu a um aumento na resistência à vancomicina (Houlihan & Russell, 2003). Os aditivos ionóforos, muito empregados na alimentação de ruminantes por reduzirem a produção de metano e amônia e aumentarem a eficiência alimentar, estão proibidos na União Europeia desde janeiro de 2006 (Official Journal of the European Union, 2003).

Devido a estes fatores, muitas pesquisas são realizadas com o intuito de desenvolver um 'aditivo natural' em substituição ao uso de outros convencionais e, a própolis, pelas suas reconhecidas propriedades biológicas (com destaque para a atividade antimicrobiana), é muito estudada na nutrição dos animais domésticos, principalmente ruminantes, com o objetivo de melhorar o desempenho produtivo desses animais.

Desde o início das investigações com própolis, vários pesquisadores estudaram suas propriedades antimicrobianas. Segundo Sforcin & Bankova (2011), *in vitro*, a

própolis pode atuar directamente sobre os microrganismos e, *in vivo*, pode estimular o sistema imunológico, ativando mecanismos envolvidos na inibição dos microrganismos. Pinto et al. (2001) observaram sensibilidade *in vitro* de amostras de bactérias Gram-positivas e Gram-negativas, isoladas do leite de vacas com mastite, à diferentes extratos de própolis, sendo que a própolis deteve maior poder antibacteriano sobre as espécies Gram-positivas do que sobre as Gram-negativas. Entretanto, o mecanismo de ação antimicrobiana da própolis ainda não foi totalmente elucidado. Muitos trabalhos relatam que as bactérias Gram-negativas são menos sensíveis à própolis (Grange e Davey 1990; Bonvehi et al. 1994 ; Vargas et al., 2004), porém, Mirzoeva et al. (1997) constataram que a própolis apresentou um potente efeito bactericida contra bactérias Gram-negativas *Rhodobacter sphaeroides*, sugerindo que a função de "barreira" da membrana externa das bactérias Gram-negativas é espécie-dependente. Os autores verificaram efeito da própolis e de alguns de seus componentes sobre a permeabilidade iônica da membrana interna das bactérias, causando dissipação do potencial de membrana. Os autores concluíram que, componentes puros da própolis, incluindo os derivados do ácido cinâmico (como ácido cafeico e CAPE) e os flavonoides (quercetina e naringenina) afetaram o potencial de membrana e motilidade bacteriana, sugerindo que estes compostos sejam componentes antimicrobianos da própolis.

Oliveira et al. (2004) avaliaram a fermentação da proteína de três fontes de nitrogênio (tripticase, farelo de soja e farinha de peixe) com ou sem monensina ou extrato de própolis e verificaram que, tanto a monensina quanto a própolis, reduziram a produção de amônia (NH_3) nos tratamentos contendo tripticase e farelo de soja, sendo que a própolis foi mais eficiente em manter maiores concentrações de proteína solúvel no início das incubações, pela redução da atividade de desaminação. Posteriormente, Oliveira et al. (2006) estudaram os efeitos *in vitro* dos inibidores monensina e própolis sobre a fermentação ruminal de aminoácidos e observaram que própolis mostrou ser mais eficiente do que a monensina em reduzir a produção de NH_3 de culturas de microrganismos ruminais em meio contendo caseína hidrolisada. Os autores também verificaram que a produção de NH_3 normalizou assim que a monensina foi removida do meio de cultura, enquanto para o tratamento com própolis, a produção se manteve em níveis baixos mesmo quando removida do meio de cultura.

Stradiotti Júnior et al. (2004a) determinaram a ação *in vitro* da própolis sobre a atividade específica de produção de NH_3 (AEPA) e sobre a fermentação ruminal em

bovinos recebendo dietas contendo 35% de concentrado e constataram que a própolis foi eficiente em inibir a AEPA pelos microrganismos ruminais, tanto *in vitro* quanto *in vivo*. Em outro experimento, Stradiotti Júnior et al. (2004b) avaliaram, *in vitro,* a eficiência do extrato de própolis em inibir a produção de gases oriundos da fermentação ruminal de diferentes alimentos e verificaram que a própolis, em relação ao controle, reduziu a produção final total e a produção final de gases para carboidratos fibrosos. Os autores também observaram que a taxa de digestão específica para carboidratos fibrosos e carboidratos não fibrosos foi superior quando se utilizou o extrato de própolis, podendo inferir que a própolis estimulou o crescimento microbiano.

Outros trabalhos, recentemente publicados, também constataram a capacidade da própolis em reduzir a produção de NH_3. Ozturk et al. (2010) investigaram, *in vitro*, os efeitos de duas diferentes concentrações de extratos etanólicos de própolis (20 e 60%) sobre a fermentação ruminal e verificaram que a concentração de nitrogênio amoniacal no fluido ruminal foi reduzida de maneira dose dependente. Do mesmo modo, Oeztuerk et al. (2010) estudaram os efeitos da nisina e própolis sobre a fermentação *in vitro* de uma dieta contendo 40% de concentrado e observaram que tanto a nisina quanto a própolis reduziram a produção de NH_3. Tais resultados indicam que a própolis pode ser uma alternativa eficiente em reduzir a produção de NH_3 ruminal e melhorar a utilização de nitrogênio (N) pelos ruminantes.

Em um estudo com cabras leiteiras, Lana et al. (2005) verificaram os efeitos da adição de óleo de soja e/ou de extrato etanólico de própolis sobre o consumo, digestibilidade de nutrientes, produção e composição do leite e alguns parâmetros de fermentação ruminal. Observou-se que o óleo de soja reduziu os consumos de matéria seca (MS) e de fibra em detergente neutro (FDN) na presença do extrato de própolis e aumentou o consumo de proteína bruta (PB) na ausência de própolis. Verificaram também o aumento nos teores de gordura, proteína e sólidos totais no leite de cabras, o aumento do pH e a redução na razão acetato:propionato no líquido ruminal para a dieta contendo óleo de soja, que se mostrou mais efetivo em alterar as variáveis analisadas do que o extrato de própolis. Lana et al. (2007) também avaliaram a inclusão de níveis crescentes de óleo de soja, extrato etanólico de própolis e própolis bruta moída na alimentação de cabras leiteiras sobre o consumo e alguns parâmetros de fermentação ruminal e não verificaram efeito dos tratamentos sobre os parâmetros avaliados.

Existem, na literatura, poucos trabalhos que relatam o uso da própolis na alimentação de vacas leiteiras. Stelzer et al. (2009) avaliaram o desempenho de vacas leiteiras consumindo rações com dois níveis de concentrado, associados ou não a um extrato etanólico de própolis sobre o consumo e a digestibilidade da MS e nutrientes, a produção e composição do leite, a eficiência alimentar e a eficiência da utilização do concentrado e verificaram nenhum efeito da própolis sobre esses parâmetros. Já Freitas et al. (2009), verificaram o efeito da adição do extrato etanólico de própolis, na alimentação de vacas da raça Holandesa, sobre a produção de leite, produção de leite corrigida para 4% de gordura, teores de gordura e proteína do leite, contagem de células somáticas e consumo de MS, sendo que a própolis aumentou a produção e o teor de proteína do leite.

Como citado anteriormente, para que os compostos ativos da própolis sejam liberados, é necessária sua extração através de solventes, como etanol, metanol e/ou água. Entretanto, dependendo do tipo e quantidade de solvente utilizado, assim como da quantidade de própolis bruta para a extração, os extratos poderão apresentar diferentes composições químicas e, consequentemente, diferentes propriedades biológicas. Com o intuito de minimizar estas variações observadas nos extratos de própolis, pesquisadores dos Departamentos de Zootecnia e Farmácia e Farmacologia da Universidade Estadual de Maringá (UEM), desenvolveram 12 produtos à base de própolis (LLOS), os quais possuem concentrações de própolis (A, B, C e D) e teores alcoólicos crescentes (1, 2 e 3), sendo estes utilizados em pesquisas envolvendo a nutrição de ruminantes.

Prado et al. (2010a) avaliaram os 12 produtos LLOS e a monensina sódica sobre a digestibilidade *in vitro* da MS (D*IV*MS) de dietas contendo 50% de volumoso e 100% volumoso. Os maiores coeficientes de digestibilidade, para a dieta contendo 50% de concentrado, foram obtidos com os LLOS C1, D1 A2 e C3 enquanto, para a dieta contendo 100% de volumoso, os maiores coeficientes foram obtidos com os LLOS B3 e C1. Para as rações contendo 50% ou 100% de volumoso, foram observados valores superiores ou semelhantes às dietas monensina e controle. Segundo os autores, a liberação da substância ativa atuante no aumento dos valores da D*IV*MS não é diretamente proporcional ao aumento da concentração de própolis e teor alcoólico, provavelmente pelas diferentes interações que ocorrem entre eles, o que influencia na extração dos compostos fenólicos. Em outro experimento, Prado et al. (2010b) estudaram os efeitos da utilização dos LLOS B1 e C3 e da monensina sódica em dieta à

base de forragem sobre o consumo, digestibilidade total (DT) e parcial e características ruminais em bovinos. Os autores verificaram que o fornecimento dos aditivos, principalmente própolis, reduziu a DT da MS, proteína bruta e NDT e a DT da FDN foi semelhante entre aditivos e menor nos animais do tratamento controle. A inclusão da própolis também refletiu em menor pH ruminal e maiores produções de acetato e ácidos graxos voláteis (AGV) totais, além de ser mais efetiva em aumentar o fluxo de PB para os intestinos, enquanto a monensina propiciou a menor razão acetato:propionato. Posteriormente, Prado et al. (2010c) testaram os LLOS B1, LLOS C3 e monensina em bubalinos que receberam dietas à base de forragem e observaram que o LLOS C1 foi superior à monensina, propiciando maior concentração de energia digestível aparente e promoveu aumento nos fluxos de PB nos intestinos. A monensina e os produtos LLOS também aumentaram a DT e intestinal dos componentes nutritivos.

O produto LLOS C1 também foi testado em bovinos terminados em confinamento, entretanto, com dosagem superior às anteriormente utilizadas (dobro da dose). Valero (2010) avaliou o efeito da monensina sódica e do LLOS C1 (com dosagem dobrada) sobre o desempenho, ingestão, digestibilidade de nutrientes e eficiência de síntese microbiana e verificou que a adição de própolis aumentou a digestibilidade do extrato etéreo em relação às dietas monensina e controle. O mesmo não foi observado por Aguiar et al. (2012) que avaliaram o produto LLOS C1, em duas dosagens diferentes, em bovinos não castrados também terminados em confinamento, sendo que não houve efeito da adição da própolis sobre os mesmos parâmetros estudados por Valero (2010).

Dando continuidade aos estudos com o produto LLOS C1, Daniel (2011) avaliou doses crescentes deste produto sobre a digestibilidade, parâmetros ruminais e eficiência de síntese microbiana em bovinos confinados e verificou nenhum efeito das diferentes concentrações do LLOS C1 sobre as variáveis estudadas. Simioni (2011) estudou o fornecimento do LLOS B1 (dobro da dose), do LLOS C1 (em duas dosagens crescentes) e da monensina sobre a digestibilidade, parâmetros ruminais, cinética de fluidos e sólidos no rúmen e produção microbiana em bovinos. Verificou-se que os produtos LLOS não alteraram a ingestão e digestibilidade dos nutrientes em relação à dieta controle, porém, em relação à monensina, o LLOS B1 resultou em menor ingestão de energia digestível, em função da menor DT da MS e do extrato etéreo; e menor digestibilidade da PB foi observada para o LLOS C1 na maior dosagem. A razão

acetato:propionato foi semelhante entre o LLOS C1 (maior dosagem), monensina e controle, no entanto, a monensina apresentou menor razão em relação ao LLOS B1 e LLOS C1 (menor dosagem). Os autores não verificaram efeito dos aditivos sobre a cinética de sólidos no trato digestível e a eficiência de síntese microbiana.

Todos estes resultados mostram que o conhecimento da quantidade dos compostos ativos presentes nos extratos de própolis, assim como de sua composição química, é imprescindível, uma vez que os efeitos observados com o uso própolis estão diretamente relacionados a estes fatores. Um grande número de trabalhos que lidam com diferentes aspectos das propriedades biológicas da própolis tem sido publicado nas últimas décadas. No entanto, de acordo com Sforcin & Bankova (2011), uma parte considerável destes trabalhos é de uso limitado, embora eles relatem atividade "forte", "notável" ou "significativa" da própolis. A razão desta limitação é a falta de comparação e avaliação científica dos resultados, pois estes geralmente não se referem à natureza química das amostras de própolis avaliadas. Estes estudos somente reportam que os testes foram realizados com "extratos de própolis"; entretanto, é importante salientar que não existe o termo "própolis". Embora de origem vegetal, a própolis é um produto apícola e, em diferentes ecossistemas, as abelhas a coletam de diferentes plantas, escolhendo representantes adequados da flora local. Por exemplo, a própolis verde brasileira é derivada, principalmente, do alecrim-do-campo (*Baccharis dracunculifolia*). O termo "própolis" não tem uma conotação química, ao contrário do nome científico de uma espécie vegetal. Uma espécie de planta é caracterizada pelo seu genoma e este genoma eventualmente determina os metabólitos secundários sintetizados pelas enzimas destas plantas, sendo estes metabólitos responsáveis pelas atividades biológicas de uma determinada espécie. No caso da própolis, ela também contém metabólitos secundários, mas estes não são os mesmos em todo o mundo, o que impossibilita a sua padronização, mas não a sua utilização. Portanto, fica claro a importância de se conhecer a quantidade e composição dos compostos ativos presentes nos diferentes extratos de própolis, a fim de relacioná-las aos efeitos observados nos estudos zootécnicos realizados *in vitro* e *in vivo* e, assim, melhor interpretá-los.

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OBJETIVOS GERAIS

1) Desenvolver e validar um método para a separação e quantificação de doze extratos à base de própolis através de CLAE e verificar se as diferentes condições de extração influenciam a composição fenólica dos extratos;
2) Caracterizar os compostos fenólicos presentes nos extratos de própolis;
3) Avaliar os efeitos de produtos à base de própolis sobre a digestibilidade, parâmetros ruminais e sanguíneos e eficiência de síntese microbiana quando adicionados às dietas de vacas leiteiras;
4) Avaliar os efeitos de produtos à base de própolis sobre a composição em ácidos graxos e capacidade antioxidante no leite de vacas;
5) Avaliar a atividade antimicrobiana de diferentes extratos de própolis sobre bactérias do rúmen.

CAPÍTULO II

(Normas: Food Chemistry)

Validation of an HPLC quantification method for phenolic acids and flavonoids in different Brazilian propolis extracts

Abstract: The objective was to develop and validate a method for separation and quantification of phenolic compounds (flavonoids and phenolic acids) in twelve propolis extracts obtained by different extraction conditions using HPLC. The validation using the apigenin standard demonstrated that the method presents linearity (correlation coefficient = 0.9993), precision (relative standard deviation <5%) and accuracy (mean recovery = 99.63%) in the concentration range 15.55 – 124.4 µg/mL. The limit of detection was 2.10 µg/mL and the limit of quantification was 6.99 µg/mL. The developed method was validated providing certainty in the quantification of total flavonoids and phenolic acids present in the different propolis extracts. The different concentrations of propolis and alcohol levels interfere in the extraction of the active compounds of propolis, which may change its chemical composition and biological properties.

Keywords: propolis, Artepillin C, flavonoids, phenolic acids, HPLC, validation

1. Introduction

Propolis is formed by a balsamic and resinous material collected by bees from the branches, flowers, pollen, buds and tree exudates. Besides these, in the hive, the bees add salivary secretions and enzymes (Pereira, Seixas & Aquino Neto, 2002). The composition of propolis is highly variable. Different substances are often being found in samples from sources in close proximity, or even in the same apiary, and its chemical composition is very complex and varied, which is closely related to the flora ecology of each region visited by bees (Lustosa, Galindo, Nunes, Randau & Rolim Neto, 2008; Salatino, Fernandes-Silva, Righi & Salatino, 2011).

Propolis is considered one of the most heterogeneous mixtures found in nature. More than 300 constituents have been identified and/or characterized in different propolis samples (Burdock, 1998). The main chemical compounds isolated from propolis until now are arranged in some major groups, i.e. aliphatic acids and esters, aromatic acids and esters, sugars, alcohols, aldehydes, fatty acids, amino acids, steroids, ketones, flavonoids (flavones, flavonols, flavanones and chalcones), terpenoids, proteins, vitamins B1, B2, B6, C, E and minerals (Menezes, 2005).

It is known that propolis has several biological properties: antimicrobial, antioxidant, antiinflammatory, immunomodulatory, hypotensive, healing, anesthetic, anti-carcinogenic, anti-HIV and anticariogenic (Bankova, Popov & Marekov, 1989; Park & Ikegaki, 1998; Park, Ikegaki, Alencar & Moura, 2000; Isla, Nieva Moreno, Sampietro & Vattuone, 2001). These activities are mainly attributed to the presence of flavonoids, that represent one of the largest and most studied classes of phenylpropanoid-derived plant specialized metabolites (Dixon & Pasinetti, 2010) and some phenolic acids in propolis, such as Artepillin C. For this reason, propolis is widely used as a popular remedy in folk medicine, in apitherapy, as a constituent of "biocosmetics", "health food" and for numerous further purposes (Bankova, de Castro & Marcucci, 2000). This points out the interest in quantifying these constituents in propolis preparations, as well as validation of analytical methodologies (Bruschi, Franco & Gremião, 2003).

The biological potential of propolis is due to a synergism that occurs between the many constituents of the compound (Marcucci, 1996). However, when obtained in the extract form, the extraction conditions can influence the composition of the extract and, consequently, its pharmacological action, reinforcing the need for standardization of methods for extracting propolis compounds.

In this context, high-performance liquid chromatography (HPLC) methods have been reported to quantify flavonoids singly or in complex biological matrices, such as herbal raw materials and extractive preparations (Bruschi, Franco & Gremião, 2003), however, the choice of an analytical method that is effective in the quantification of such compounds is essential. Also, working with standardized material will allow scientists to connect a particular chemical propolis type to a specific type of biological activity and formulate recommendations for mainstream practitioners (Bankova, 2005).

Therefore, the objective was to develop and validate a method for separation and quantification of phenolic compounds (flavonoids and phenolic acids) in Brazilian propolis extracts obtained by different extraction conditions using HPLC.

2. Material and methods

2.1. Material

The propolis samples were obtained from the apiary of the Experimental Farm of Iguatemi (FEI), belonging to the Universidade Estadual de Maringá, Paraná State, Brazil, being certified as organic. The apiary is located within a reserve of eucalyptus (*Eucalyptus* sp.) surrounded by native forest, with the presence of alecrim-do-campo (*Baccharis dracunculifolia*). The propolis samples were obtained from colonies of africanized honeybees (*Apis mellifera*) and were placed in plastic containers and stored at a freezing temperature of -22^{o}C.

2.2. Preparation of the propolis dry extracts

The dry extracts were obtained at increasing concentrations of propolis between 5.0 and 30.0% (w/v) in water-alcohol solutions between 60.0 and 93.8% (v/v) of alcohol by turbo extraction, for 15 minutes. The extracts were filtered under vacuum and subjected to the dealcoholisation in a rotary evaporator (Buchi, model RT 210) to the limit of 15% of alcohol. Then, they were subjected to a spray drying process (nebulizer Labmaq, model MSD 1.0 with capacity for 1 L/hour), with inlet temperature of 100^{o}C. After drying, they were stored in closed bottles and kept at frozen storage (-22°C). The propolis extracts were obtained using four increasing concentrations of propolis (A, B, C and D) each with three increasing alcohol levels (1, 2 and 3) resulting in a total number of twelve different propolis dry extracts, named A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2 and D3, and registered in the National Institute of Industrial Property – Brazil, under n^{o}. 0605768-3.

2.3. Content of the active substances quantified by HPLC

2.3.1. Preparation of the extraction solutions

Seven milligrams of each propolis dry extract was dissolved in 15 mL of ultrapure deionized water (Milli-Q system, Millipore, Billerica, USA). After that, it was transferred to a separation funnel and about 25 mL of ethyl acetate were added for the extraction. The obtained solution was transferred to a beaker containing anhydrous sodium sulfate, to remove any traces of water from the solution. After that, the salt was removed and the solution was left in a water bath (70^{o}C) for complete evaporation of the ethyl acetate. The dry residue obtained was resuspended in methanol (5 mL) and filtered through a polyvinylidene fluoride (PVDF) membrane-HV Durapore, with 0.45 µm and 13 mm in diameter (Millipore, lot number B8PN70633, Billerica, USA) obtaining, thereby, the sample to be analyzed in the chromatograph.

2.3.2. Calibration curves

The quantification of flavonoids was performed using calibration curves obtained from apigenin, chrysin and naringenin standards, while for phenolic acids, *p*-coumaric acid and 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) standards were used. The chrysin standard (Chrysin 97%, lot number S36906-269, Sigma-Aldrich Co., St. Louis, USA) was dissolved in 5.0 mL of methanol (Methanol Baker HPLC Solvent, Mallinckrodt Baker Inc., Philipsburg, USA) obtaining the following concentrations: 198.0, 99.0, 66.0, 49.5, 33.0, 24.75 and 19.80 µg/mL. The apigenin standard (approximately 95%, lot number 47H2505, Sigma-Aldrich Co., St. Louis, USA) was dissolved in 5 mL of methanol resulting in the following concentrations: 15.55, 20.73, 31.10, 41.46, 93.30 and 124.4 µg/mL. The naringenin standard (approximately 95%, lot number 118K1468, Sigma-Aldrich Co., St. Louis, USA) was dissolved in 5 mL of methanol, resulting in the following concentrations: 17.96, 22.45, 29.93; 44.90, 59.86 and 179.60 µg/mL.

The Artepillin C standard (“Artepillin C from propolis”, 98%, lot number STN0051, Wako Pure Chemical Industries, Osaka, Japan) was dissolved in 5.0 mL of methanol, obtaining the following concentrations: 10.96, 13.70, 18.26, 27.40, 36.53 and 54.80 µg/mL. The *p*-coumaric acid standard (lot number 00003833-KEC, ChromaDex, Irvine, USA) was dissolved in 5 mL of methanol, resulting in the following concentrations: 12.91, 15.06, 18.08, 22.60, 30.13 and 90.40 µg/mL. The standard solutions obtained were filtered through a PVDF membrane-HV Durapore. For each

standard concentration, there were five replicates and the calibration curves were calculated using linear regression.

2.3.3. Chromatographic conditions

An Alliance HPLC-PDA system, which consisted of a Waters e2695 separation module (Waters Co., Mildford, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, and a Waters 2996 photodiode array detector of 512 photodiodes and an optical resolution of 1.2 nm, was used for the chromatographic analysis. Data were collected and integrated with the Waters Empower2 software (Waters Co., Mildford, USA). A linear gradient (Table 1) was used as mobile phase and, as a stationary phase, two columns were used for the optimization of the chromatographic conditions: a Phenomenex Luna PFP(2) (pentafluorophenyl) column, with 5µ particles, 100 Å, 250 x 4.6 mm and a guard column with the same material and porosity, and a HiCHROM® RP C_{18} 4.6 x 25mm column, filled with Hichrom 100 and 5µ particles.

Table 1

Linear gradient system used as mobile phase

Time (minutes)	Methanol (%)[a]
0.00	55
5.00	58
7.50	62
25.00	85
30.00	100

[a] Aqueous phase containing acetic acid 5.0% or acetonitrile 2.5% (v/v).

With the same purpose, two mobile phases were used: an aqueous solution containing acetic acid 5.0% (v/v) and methanol, and another aqueous solution containing acetonitrile 2.5% (v/v) and methanol, using a flow rate of 1.0 mL/min. The detection was performed at 310 nm and the running time was 40 minutes. For the analysis, aliquots of 20 µL were used, with five replicates for each extract analyzed. The results were expressed as mg/g of propolis dry extract.

2.4. Validation of analytical method

To validate the analytical method, the recommendations outlined in the guidelines prepared by the National Institute of Metrology, Quality and Technology (INMETRO, 2010), National Health Surveillance Agency (ANVISA, 2003) and International Conference on Harmonisation (ICH, 2005) were followed.

2.4.1. Linearity

The linearity of the calibration curve was determined by chromatographic analysis (HPLC) using different concentrations of the apigenin standard (described above). Five determinations were carried out for each solution. The calibration curves were obtained by plotting the peak area of apigenin versus the concentration of the standard solutions.

2.4.2. Precision (repeatability, intermediate precision and reproducibility)

The precision was determined through a three points curve, with five repetitions. The repeatability of the method was evaluated on the same day, while the intermediate precision was determined in four non-consecutive days. The standard solution was analyzed at three concentration levels (15.55, 41.46 and 124.4 µg/mL) for repeatability and at 127.6 µg/mL for intermediate precision. Five determinations were carried out for each solution. The precision was expressed as relative standard deviation (RSD, %) of apigenin concentrations.

To determine intra-laboratory repeatability, a Shimadzu liquid chromatograph, model 20A, with a UV/vis detector (SPD-20A), equipped with a quaternary pump (LC-20 AT) and Rheodyne manual injector with 20µL sample injection loop (Shimadzu Co., Tokyo, Japan) was used. The mobile and stationary phases were the same used in this experiment. For repeatability determination, a standard solution of apigenin (127.6 µg/mL), with five replications, was prepared.

For reproducibility, the same Shimadzu liquid chromatograph, mentioned above, was used and the mobile and stationary phases were also the same. However, the apigenin standard solution was injected by a different analyst, although at the same concentration as used for repeatability, with five replications.

2.4.3. Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curve of the apigenin standard. LOD was calculated according to the expression $3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. LOQ was established by using the expression $10\sigma/S$.

2.4.4. Accuracy

The accuracy was evaluated with the recovery test by analyzing the mixture prepared by adding the solution of apigenin in three concentration levels (15.55, 41.46 and 124.4 µg/mL) to the propolis extract containing a known amount of the analyte. Five determinations were performed for each solution. The percentage recovery was calculated by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100.

2.4.5. Stability of the analyte

A test to check the stability of the analyte, with the apigenin standard for this purpose, was conducted. Approximately 638.0 µg of the apigenin standard were weighed, dissolved in 5 mL of methanol and filtered through a PVDF membrane-HV Durapore. A sample of the solution was kept at 5°C, while another sample was kept at room temperature, protected from light. The apigenin solution was injected into HPLC immediately after preparation, with five replicates per studied period. Every seven days, in a period of 21 days, the two solutions (refrigerated and room temperature) were injected under the same chromatographic conditions.

2.5. Statistical analysis

For the quantification of phenolic compounds in the propolis extracts, the data were analyzed by SAS software (SAS, 2001), using a 4^3 factorial arrangement. Differences between treatment means were determined by Tukey test. Tests that had p-values<0.01 were considered statistically significant. To validate the values obtained from the analytical curve of apigenin, the data were subjected to analysis of variance (ANOVA) and the F-value was calculated for linear regression and residue. For these calculations, the Statistica software (Statsoft Inc., 2004) was used.

3. Results and discussion

To quantify the flavonoids in propolis, three standards were chosen (apigenin, chrysin and naringenin), because they are constituents of propolis. For phenolic acids, Artepillin C, the main phenolic acid found in Brazilian propolis (Estrada, Silva & Antunes, 2008), was chosen as well as *p*-coumaric acid, which is present in high concentrations in the extracts studied and show absorptivity very close to others phenolic acids of propolis, such as cinnamic acid, chlorogenic acid, benzoic acid and its derivatives. This group of substances also presents a very close retention time, between 01 -10 minutes. New chromatographic conditions were established due to circumstances of laboratory work and, therefore, proceeded to the optimization of new conditions, as described below.

3.1. Optimization of chromatographic conditions

The chromatographic conditions used in the experiment showed good results. The pentafluorophenyl column (Phenomenex Luna PFP(2)) was chosen because it was more efficient in the separation of the propolis compounds when compared to the reverse phase column (HiCHROM® RP C_{18}). Regarding the mobile phase, the linear gradient system using acetic acid 5.0% (v/v) obtained results as good as the mobile phase containing acetonitrile 2.5% (v/v). Despite the good results using acetonitrile, the acetic acid 5.0% was chosen as mobile phase, due to its low cost compared to acetonitrile. The initial running time was 40 minutes; however, it was observed that 30 minutes were enough for the chromatographic analysis. The wavelength of 310 nm was chosen as the most appropriate for reading the compounds in the propolis extracts, especially flavonoids. The results with the optimized condition showed good selectivity of the method, which is the ability of a method to accurately quantify the analyte in the presence of interference in the sample (Paschoal, Rath, Airoldi & Reyes, 2008).

3.2. Validation of the analytical method

3.2.1. Linearity

The results obtained in the linearity study showed that the analytical curve for apigenin was linear, in the range 15.55-124.4 µg/mL, as shown in Table 2.

Table 2

Parameters of linearity for the calibration curve of the apigenin standard

Standard	Linearity range	Slope (*a*)	Intercept (*b*)	RSD(%)[a]	r^{2}[b]	r[c]
Apigenin	15.55 – 124.4 µg/mL	5.97e+4	-6.21e+4	2.84	0.9987	0.9993

[a] Relative standard deviation.
[b] Coefficient of determination.
[c] Correlation coefficient.

The correlation coefficient (*r*) was 0.9993, showing that there is linear correlation between the area and the concentration of apigenin. This value is consistent with the resolution RE n° 899 (ANVISA, 2003), establishing *r*=0.99 as minimum acceptable criteria. According to Leite (1998), the closer of the unit the value is, the greater the relationship between *X* (concentration) and *Y* (area), with a higher probability that there is a defined linear relation.

According to INMETRO (2010), a method is considered sensitive when small variations in concentration result in great variation in the response, i.e., greater slope. The results obtained with apigenin showed slope of 5.97e+4, which can be considered an expressive value.

A common mistake is to use the coefficient of determination (r^2) (Table 2) to evaluate a model and, conclude, if this value is sufficiently high, that the fit is satisfactory. A value of 0.9987 for r^2, for example, is obviously high, but only means that 99.87% of total variation about the mean was explained by the model. It is possible that the 0.13% is concentrated in a single portion of the curve, which indicates lack of fit. Thus, the right way to do the interpretation is through the F-test to assess the lack of fit (Pimentel & Barros Neto, 1996). Therefore, applying the F-test, the results obtained from the fit tests of the linear model and regression validity showed that the regression equation was statistically significant. This ratio showed F-value of 0.0030, which is lower than the tabulated critical value (2.78) with 28 degrees of freedom and 95% confidence. Therefore, as the condition F-calculated <F-critical was answered, the linearity was confirmed, i.e., the linear model presented well suited in the concentration range studied (Chui, Zucchini & Lichtig, 2001).

3.2.2. Precision (repeatability, intermediate precision and reproducibility)

For the repeatability tests, three different concentrations of apigenin were used. For concentrations of 15.55, 41.46 and 124.4 µg/mL the following RSD were achieved: 1.27, 0.30 and 0.23%. The intermediate precision showed RSD values of 3.59%. According to ANVISA (2003), RSD values higher than 5% are not acceptable, so it can be stated that the results obtained in this experiment are accurate, since they are below the acceptable limit, which also shows the degree of reliability of the method. The RSD value obtained for the intra-laboratory repeatability was 2.20%, which is within the limit considered acceptable. As for the reproducibility, the RSD value was 1.28%, also within the acceptable limit.

3.2.3. Limit of detection and limit of quantification

LOD is understood to be the smallest amount or analyte concentration in the sample that can be distinguished, reliably, from scratch or from background noise (Paschoal et al., 2008). The LOD obtained under the experimental conditions was 2.10 µg/mL. The LOQ under the evaluated conditions was 6.99 µg/mL, which is defined as the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the established experimental conditions (ANVISA, 2003).

3.2.4. Accuracy

The accuracy of an analytical method is the closeness of the results obtained by the method under study in relation to the true value (ANVISA, 2003). The data recorded in the recovery test of apigenin, obtained in the evaluated propolis extracts for the concentrations 15.55, 41.46 and 124.40 showed the following recoveries: 93.63, 106.14 and 99.14% respectively, with average recovery of 99.63% and RSD of 6.27%. This value confirms the accuracy of the analytical method.

3.2.5. Stability of the analyte

The evaluation of the stability of the solutions used in the analytical method is of great importance, as degradation of the analyte or matrix constituents, during storage or analysis of the sample, can affect the accuracy of the results (Paschoal et al., 2008). The table below (Table 3) is a comparison of the obtained results, in the period of 21 days,

for the apigenin solutions injected into the HPLC under two storage conditions (room temperature (21°C) and refrigerated (4°C)).

Table 3

Areas obtained for the apigenin samples injected into HPLC with different injection days after preparation

Days after preparation	Apigenin (AU[a])			
	Room temperature	RSD,%	Refrigerated	RSD,%
0 day[b]	6931543		6931543	
7 days	7100984	1.70	7079002	1.48
14 days	7886163	9.11	8020450	10.29
21 days	8017464	10.27	8132519	11.27

[a] Absorbance units.
[b] The sample was injected immediately after preparation.
For each sample $n = 5$.

No major changes were observed in the analyte for the evaluation period (21 days), both for the sample kept refrigerated and for the sample kept at room temperature. However, greater stability of the analyte was observed for the first 7 days of preparation for both samples, while after 7 days the samples began to degrade.

According to Lanças (2004), the criterion for acceptance of a stable solution is a variation below 10% in the concentration of the analyte of interest compared to the concentration at time zero (day of preparation). Therefore, the samples were considered stable for a period of 14 days. In addition, the recently prepared solutions, as those kept under refrigerated conditions (4°C) showed the same chromatographic profile and no degradation products were observed.

3.3. Quantification of the propolis extracts

An interaction of the concentration of propolis and the alcohol level in the estimation of total flavonoids quantified in apigenin ($p<0.01$, Table 4) was observed. These results are in agreement with Cottica, Sawaya, Eberlin, Franco, Zeoula & Visentainer (2011a) which also reported an interaction between concentration of propolis and alcohol level for the content of total flavonoids quantified in quercetin.

Table 4

Average concentration of flavonoids (mg/g) in propolis dry extracts[a] quantified in apigenin

	Concentration of propolis			
	A	B	C	D
Alcohol level 1	100.18^{Bc}	109.17^{Aa}	79.16^{Cb}	31.54^{Dc}
Alcohol level 2	122.81^{Ab}	67.33^{Cb}	88.16^{Ba}	57.73^{Da}
Alcohol level 3	129.17^{Aa}	60.35^{Bc}	44.52^{Dc}	54.95^{Cb}

Means followed by different letters, uppercase in the same line and lowercase in the same column, are statistically different ($p<0.01$) by Tukey test. For each sample $n = 5$. [a] Concentrations of propolis (A, B, C, D) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1, 2, 3) between 60.0 and 93.8% (v/v) of alcohol.

For the lowest alcohol level (1), the optimal concentration of propolis was B (third highest concentration), because it allowed greater extraction of flavonoids (109.17 mg/g). However, for intermediate (2) and higher (3) alcohol levels the optimal concentration of propolis was A (lowest concentration), which resulted in higher ($p<0.01$) extraction of flavonoids, with means of 122.81 and 129.17 mg/g, respectively.

In relation to the concentrations of propolis was found that, for the lowest concentration (A), the increase in alcohol level improved the extraction of flavonoids (from 100.18 to 129.17 mg/g). Though, for the concentration of propolis B the opposite was observed, the increase in the alcohol level was reflected in lower extraction for flavonoids (from 109.17 to 60.35 mg/g). These results indicate that, in the first case (concentration A), the lower alcohol level was insufficient to extract flavonoids but, for concentration of propolis B (concentration B>A), when the alcohol level was increased there was lower extraction of flavonoids, and this was probably due to greater removal of resin and/or wax present in the sample, which may have an adverse effect on the extraction of flavonoids, as observed by Prado, Zeoula, Pontara, Franco, Novello & Geron (2010).

These observations corroborate with the observed values for the content of flavonoids extracted at the concentrations C and D, which are below those extracted in the concentrations A and B. However, differences in the concentrations of propolis and alcohol levels used for the extraction of the active compounds of propolis resulted in extracts with different compositions.

Among the concentrations of propolis (5.0 to 30.0% (w/v)) and alcohol content (50.0 to 93.8% (v/v)) tested in this work, the interaction between the highest

concentration of propolis D (30.0%) and lower alcohol content 1 (50.0%) resulted in lower extraction of active compounds than for higher alcohol levels (Table 4), which may be due to the low alcohol content used, that was insufficient for an efficient extraction of flavonoids in higher concentrations of propolis.

In general, the lowest concentration of propolis (A) provided the highest extraction of flavonoids. However, the yield of the extracts, regardless the alcohol levels, is very low, which can derail production on industrial scale and, consequently, its commercial use. In this context, the extract B1 was elected as the most suitable, because it contains high levels of flavonoids and provides good performance in its production.

One of the objectives proposed for the production of the propolis dry extracts was its use in ruminant nutrition, in order to assess their effects on rumen microbial and antioxidant activity metabolisms and, consequently, the effects of propolis in food produced from these animals (meat and milk). In *in vivo* studies (De Aguiar et al., 2011; Cottica et al., 2011b), it was observed that the extract B1 improved milk quality of dairy cows, through a significant decrease ($p<0.05$) in the n-6/n-3 ratio and increase in the antioxidant activity ($p<0.05$) when compared to control treatment. Also, milk resulting from the addition of the extract C3 showed a significant increase in the amount of monounsaturated and n-3 fatty acids, and a decrease ($p = 0.00631$) in the amount of saturated fatty acids, which shows that the addition of the propolis extracts in dairy cows diets improves milk quality, which is more beneficial for human consumption.

It was observed that the extracts showed a chromatographic profile capable of quantification of flavonoids, regardless of the standard used. The profile of the flavonoids found in the propolis dry extracts shows that these substances have absorptivity close to naringenin, which is a flavanone. This suggests that the naringenin standard provides the most precise quantification of the flavonoids present in propolis. In numerical terms, the naringenin ($r = 0.9997$; $Y = 2.88e+4X + 3.28e+3$) was the standard that showed higher content of flavonoids, followed by chrysin ($r = 0.9999$; $Y = 5.73e+4X – 1.60e+5$) and apigenin ($r = 0.9993$; $Y = 5.97e+4X – 6.21e+4$), respectively.

A large part of the phenolic acids in propolis, when analyzed by HPLC, is grouped at the beginning of the chromatogram. In the present study, were quantified the phenolic acids grouped at the beginning of the chromatogram, as well as CAPE and Artepillin C, which are two phenolic compounds present in the retention range of flavonoids. The phenolic acids were quantified using calibration curves obtained for *p*-

coumaric acid and Artepillin C, while the sum of the phenolic acids grouped at the beginning of the chromatogram with CAPE and Artepillin C is the total phenolic acids present in the extracts analyzed (Table 5).

Table 5

Average concentration of phenolic acids and average concentration of total phenolic acids (mg/g) in propolis dry extracts[a] quantified in *p*-coumaric acid

	Concentration of propolis			
	A	B	C	D
Phenolic acids[b]				
Alcohol level 1	12.14^{Dab}	23.16^{Ca}	26.77^{Ba}	33.74^{Aa}
Alcohol level 2	12.93^{Dab}	21.04^{Bb}	18.38^{Cb}	31.60^{Ab}
Alcohol level 3	11.33^{Cb}	7.88^{Dc}	16.66^{Bc}	29.20^{Ac}
CAPE and Artepillin C				
Alcohol level 1	20.75^{Ac}	15.80^{Ba}	15.20^{Ba}	2.82^{Cc}
Alcohol level 2	28.71^{Ab}	11.26^{Dc}	12.56^{Cb}	13.23^{Ba}
Alcohol level 3	37.27^{Aa}	13.97^{Bb}	9.09^{Cc}	7.77^{Db}
Total phenolic acids[c]				
Alcohol level 1	32.89^{Dc}	38.96^{Ba}	41.97^{Aa}	36.56^{Cb}
Alcohol level 2	41.64^{Bb}	32.30^{Cb}	30.94^{Cb}	44.83^{Aa}
Alcohol level 3	48.60^{Aa}	21.85^{Dc}	25.75^{Cc}	36.97^{Bb}

Means followed by different letters, uppercase in the same line and lowercase in the same column, are statistically different ($p<0.01$) by Tukey test. For each sample $n = 5$.

[a] Concentrations of propolis (A, B, C, D) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1, 2, 3) between 60.0 and 93.8% (v/v) of alcohol.

[b] Phenolic acids quantified at the beginning of the chromatogram.

[c] Sum of the phenolic acids grouped at the beginning of the chromatogram with CAPE and Artepillin C.

Interactions ($p<0.01$) between alcohol levels and concentrations of propolis for the quantification in *p*-coumaric acid ($r = 0.9997$; $Y = 1.67e+5X + 1.68e+4$) were observed (Table 5). It appears that, for all alcohol levels, the highest extraction of phenolic acids, grouped at the beginning of the chromatogram, was for concentration D. This data suggest that higher concentrations of propolis (D) were efficient in the extraction of *p*-coumaric acid and the main phenolic acids, such as cinnamic acid, caffeic acid, chlorogenic acid, benzoic acid and its derivatives, diverging from the extraction of flavonoids, which was more efficient in lower concentrations of propolis.

For the phenolic acids CAPE and Artepillin C, interactions (p<0.01) between the concentration of propolis and alcohol level were observed (Table 5). CAPE, a caffeic acid derivative, has been widely studied due to its biological properties, among them, the antioxidant activity. Russo, Longo & Vanella (2002) investigated the antioxidant activity of propolis extracts with and without CAPE and found that the extract containing CAPE was more active than the extract without CAPE, suggesting that this substance has an important role in the antioxidant activity of propolis. Another compound with antimicrobial, anti-inflammatory, anticancer and antioxidant activities is the Artepillin C, commonly found in Brazilian propolis and with some structural similarity to CAPE (the presence of the *trans*-cinnamoyl system) (Bankova, 2009). Therefore, the highest concentration of these two constituents, CAPE and Artepillin C, justifies its quantification independently of other phenolic acids and derivatives.

It was observed that the lowest concentration of propolis (A) at all alcohol levels, was the best for the extraction of CAPE and Artepillin C and, among the twelve extracts studied, the highest content of these compounds (37.27 mg/g) occurred for the concentration of propolis A with alcohol level 3. For extracts B and C, the best extraction of CAPE and Artepillin C occurred for the lowest alcohol content (1), with means of 15.80 and 15.20 mg/g, respectively. On the other hand, this points out that the lower extraction of CAPE and Artepillin C occurred for the concentration of propolis D and alcohol level 1, similar to that observed in the quantification of flavonoids.

Regarding the total phenolic acids, interactions (p<0.01) between alcohol levels and concentrations of propolis were also observed (Table 5). The highest extraction of total phenolic acids occurred for the lowest concentration of propolis (A) and alcohol level 3, with mean of 48.60 mg/g of dry extract. On the other hand, the lowest content of total phenolic acids was 21.85 mg/g and occurred for the concentration B with alcohol level 3.

The phenolic acids were also quantified in Artepillin C (r = 0.9996; Y = 2.05e+5X – 8.25e+4) and showed a chromatographic profile capable in quantify the phenolic acids, regardless of the standard used.

The Table 6 presents the parameters derived from statistical analysis of the data studied. It is observed that the values of coefficient of determination (r^2) are close to 1.0 and the p-value was highly significant.

Table 6

Coefficient of determination (r^2), relative standard deviation (RSD) and *p*-value for the interactions occurring in the studied variables.

Variable	r^2,%	RSD,%	*p*-value
Flavonoids in apigenin	0.9993	1.28	0.00001
Flavonoids in chrysin	0.9995	1.02	0.00001
Flavonoids in naringenin	0.9989	1.80	0.00001
Phenolic acids in *p*-coumaric acid	0.9978	2.42	0.00001
CAPE and Artepillin C in *p*-coumaric acid	0.9996	1.92	0.00001
Total phenolic acids in *p*-coumaric acid	0.9947	1.92	0.00001
Phenolic acids in Artepillin C	0.9978	2.63	0.00001
CAPE and Artepillin C in Artepillin C	0.9996	1.39	0.00001
Total phenolic acids in Artepillin C	0.9948	1.97	0.00001

For flavonoids, the A3 was the most efficient in the extraction of these compounds, followed by extracts A2 and B1. For phenolic acids, the extract A3 had higher extraction, followed by extracts D2 and C1. In general, the best interaction between concentration of propolis and alcohol level for the extraction of the phenolic compounds present in Brazilian propolis occurred to extract A3, however, the yield of the extracts with lower concentration of propolis (A), regardless the alcohol levels, was very low, which is unfavorable to its production.

4. Conclusions

The technique for the quantification of propolis extracts by HPLC was validated, providing certainty in the content of phenolic compounds present in different extracts of propolis, and can be used in the quantification of propolis extracts with different purposes. However, the different concentrations of propolis and the different alcohol levels interfere in the extraction of the active compounds of propolis, which may change its chemical composition and, consequently, its biological properties.

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CAPÍTULO III

(Normas: Food Chemistry)

Phenolic fingerprint of Brazilian propolis extracts obtained by different extraction conditions using HPLC

Abstract: The objective was to obtain a phenolic fingerprint of twelve Brazilian propolis extracts obtained by different extraction conditions (concentration of propolis and alcohol level), using standards of flavonoids and phenolic acids to detect the compounds present in propolis using HPLC. Regarding the phenolic acids, four compounds were identified in the twelve extracts (caffeic acid, *p*-coumaric acid, CAPE and Artepillin C), while some compounds were detected only in some extracts (gallic acid, chlorogenic acid). For flavonoids, apigenin, pinocembrin, and chrysin were detected in all extracts and galangin and acacetin were detected in most extracts; however naringenin has been identified only in two extracts. The different concentrations of propolis and alcohol levels directly influence the extraction of some phenolic compounds, which alters the chemical composition of the propolis extracts, reinforcing the need of reliable criteria for chemical standardization of different propolis types.

Keywords: Artepillin C, chromatographic profile, flavonoids, phenolic acids, propolis extracts

1. Introduction

Propolis designates a series of gums, resins and balms of viscous consistency which are gathered by honeybees from certain parts, mainly the buds and barks of plants, especially those found on coniferous trees. Bees bring propolis back to the hive, where it is modified and mixed with other substances including the bees' own wax and salivary secretions (Russo, Longo & Vanella, 2002).

Tropical propolis, particularly from Brazil, has shown significant differences in their chemical composition compared to the propolis from the temperate zone. For this

reason, Brazilian propolis has become the object of great interest from scientists (Trusheva et al., 2006).

Many flavonoids have been identified in Brazilian propolis, *e.g.*, acacetin, pinobanksin, kampferol, apigenin, pinocembrin, chrysin, galangin, isosakuranetin and betuletol (Park, Alencar, Scamparini & Aguiar, 2002a; Park, Alencar & Aguiar, 2002b; Funari & Ferro, 2006; Chang, Piló-Veloso, Morais & Nascimento, 2008; Tavares, Lemos, Arriaga, Santiago & Braz-Filho, 2010; Cottica, Sawaya, Eberlin, Franco, Zeoula & Visentainer, 2011). Among the phenolic acids, cinnamic acid and derivatives (caffeic acid, *p*-coumaric acid, drupanin, bacharin), benzoic acid, 3-prenyl-hydroxycinnamic acid, chlorogenic acid, ferulic acid, trans-cinnamic acid, caffeic acid phenethyl ester (CAPE) and Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) are also found in Brazilian propolis (Park et al., 2002a, b; Funari & Ferro, 2006; Sousa, Furtado, Jorge, Soares & Bastos, 2007; Chang et al., 2008; Salomão et al., 2008; Tavares et al., 2010; Chikaraishi, Izuta, Shimazawa, Mishima & Hara 2010).

Recently, Artepillin C has been studied in samples of Brazilian propolis and several biological activities have been attributed to Artepillin C, such as antioxidant, antimicrobial and antitumor activities. (Estrada, Silva & Antunes, 2008).

However, propolis chemical composition depends on the phytogeographic characteristics of the site of collection, since bees choose different plants as source of propolis in different habitats (Popova, Chen, Chen, Huang & Bankova, 2010). This aspect difficults propolis standardization and different solvents (ethanol, methanol and water) may extract different compounds, influencing its activity (Cunha et al., 2004). According to Bankova (2005), an universal chemical standardization for a product as changeable as propolis is not possible, however, it is possible to formulate different propolis types according to their plant source and the corresponding chemical profile. Therefore, it is evident the need for more research with propolis, so that there is better understanding of its biological activities, mainly due to the great diversity in its composition.

Due to the possible variation in the chemical composition of the propolis extracts used in this study, the aim was to obtain a phenolic fingerprint of Brazilian propolis extracts obtained by different extraction conditions, using standards of flavonoids and phenolic acids to detect the compounds present in propolis, by high-performance liquid chromatography (HPLC).

2. Material and methods

2.1. Material

The propolis samples were obtained from the apiary of the Experimental Farm of Iguatemi (FEI), belonging to the Universidade Estadual de Maringá, Paraná State, Brazil, being certified as organic. The apiary is located within a reserve of eucalyptus (*Eucalyptus* sp.) surrounded by native forest, with the presence of alecrim-do-campo (*Baccharis dracunculifolia*). The propolis samples were obtained from colonies of africanized honeybees (*Apis mellifera*) and were placed in plastic containers and stored at a freezing temperature of -22^{o}C.

2.2. Preparation of the propolis dry extracts

The dry extracts were obtained at increasing concentrations of propolis between 5.0 and 30.0% (w/v) in water-alcohol solutions between 60.0 and 93.8% (v/v) of alcohol by turbo extraction, for 15 minutes. The extracts were filtered under vacuum and subjected to the dealcoholisation in a rotary evaporator (Buchi, model RT 210) to the limit of 15% of alcohol. Then, they were subjected to a spray drying process (nebulizer Labmaq, model MSD 1.0 with capacity for 1 L/hour), with inlet temperature of 100^{o}C. After drying, they were stored in closed bottles and kept at frozen storage (-22°C). The propolis extracts were obtained using four increasing concentrations of propolis (A, B, C and D) each with three increasing alcohol levels (1, 2 and 3) resulting in a total number of twelve different propolis dry extracts, named A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2 and D3, and registered in the National Institute of Industrial Property – Brazil, under n^{o}. 0605768-3.

2.3. Content of the active substances quantified by HPLC

2.3.1. Preparation of the extraction solutions

Seven milligrams of each propolis dry extract was dissolved in 15 mL of ultrapure deionized water (Milli-Q system, Millipore, Billerica, USA). After that, it was transferred to a separation funnel and about 25 mL of ethyl acetate were added for the extraction. The obtained solution was transferred to a beaker containing anhydrous sodium sulfate, to remove any traces of water from the solution. After that, the salt was

removed and the solution was left in a water bath (70°C) for complete evaporation of the ethyl acetate. The dry residue obtained was resuspended in methanol (5 mL) and filtered through a polyvinylidene fluoride (PVDF) membrane-HV Durapore, with 0.45 µm and 13 mm in diameter (Millipore, lot number B8PN70633, Billerica, USA) obtaining, thereby, the sample to be analyzed in the chromatograph.

2.3.2. Characterization of the compounds detected in the propolis extracts

For the characterization of the propolis extracts the following standards were used: acacetin (lot number 75H4003, Sigma-Aldrich Co., St. Louis, USA), benzoic acid (lot number BCBB3845, Sigma-Aldrich Co., St. Louis, USA), caffeic acid (pure, lot number 0001416536, Sigma-Aldrich Co., St. Louis, USA), chlorogenic acid (minimum 95%, lot number 079K0991, Sigma-Aldrich Co., St. Louis, USA), gallic acid (lot number 00007040-226, ChromaDex, Irvine, USA), *p*-coumaric acid (lot number 00003833-KEC, ChromaDex, Irvine, USA), apigenin (approximately 95%, lot number 47H2505, Sigma-Aldrich Co., St. Louis, USA), Artepillin C ("Artepillin C from propolis", 98%, lot number STN0051, Wako Pure Chemical Industries, Osaka, Japan), CAPE (lot number 00003021-974, ChromaDex, Irvine, USA), chrysin (97%, lot number S36906-269, Sigma-Aldrich Co., St. Louis, USA), galangin (lot number MKBD1031, Sigma-Aldrich Co., St. Louis, USA), naringenin (approximately 95%, lot number 118K1468, Sigma-Aldrich Co., St. Louis, USA) and pinocembrin (lot number 129K1415, Sigma-Aldrich Co., St. Louis, USA).

Stock solutions were obtained from the standards described above (700 µg/mL), which were diluted to obtain solutions with the following concentrations: acacetin 70 µg/mL, benzoic acid 233.33 µg/mL, caffeic acid 30.46 µg/mL, chlorogenic acid 77.77 µg/mL, gallic acid 350.00 µg/mL, *p*-coumaric acid 23.33 µg/mL, apigenin 50.00 µg/mL, Artepillin C 35.00 µg/mL, CAPE 35.00 µg/mL, chrysin 50.00 µg/mL, galangin 175.00 µg/mL, naringenin 70.00 µg/mL and pinocembrin 100.00 µg/mL. All solutions obtained were filtered through a polyvinylidene fluoride (PVDF) membrane-HV Durapore, with 0.45 µm and 13 mm in diameter (Millipore, lot number B8PN70633, Billerica, USA). For each standard, were performed injections of 20 µL, with five replicates for each substance. Detection was performed at 310 nm, with the exception of benzoic acid, which has improved detection at 284 nm.

2.3.3. Addition of standard to the propolis samples

To confirm the presence of the substances to be identified, equal volumes of standard solutions (item 2.3.2) were added to the samples (propolis extracts) prepared according to item 2.3.1, and the chromatographic profiles were obtained.

2.3.4. Chromatographic conditions

An Alliance HPLC-PDA system, which consisted of a Waters e2695 separation module (Waters Co., Mildford, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, and a Waters 2996 photodiode array detector of 512 photodiodes and an optical resolution of 1.2 nm, was used for the chromatographic analysis. Data were collected and integrated with the Waters Empower2 software (Waters Co., Mildford, USA). The chromatographic conditions used in this study were the same described in Chapter II.

3. Results and discussion

3.1. Chemical profile of propolis extracts obtained by HPLC

In this study, we chose to work with a wavelength of 310 nm, because this is the most appropriate for the more selective detection of flavonoids, phenolic acids and derivatives. Fig. 1 shows the chromatogram of four analyzed extracts (A1, B1, C3 and D1), obtained with different concentrations of propolis and alcohol levels. It can be observed that there are small differences in the chromatographic profile, and these differences are more evident in the intensity of detection than in chemical composition.

The determination of the chemical profile of propolis is extremely important, since their biological properties depend on their chemical composition. According to Bankova (2005), it is important for researchers studying biological activity of propolis to be aware of the existence of the problem of standardization and to be able to distinguish between different propolis types. It is essential to have detailed and reliable comparative data on every type of biological activity, combined with chemical data, in order to decide if some specific areas of application of a particular propolis type can be formulated as preferable. The biological tests have to be performed with chemically well characterized, and if possible, chemically standardized propolis.

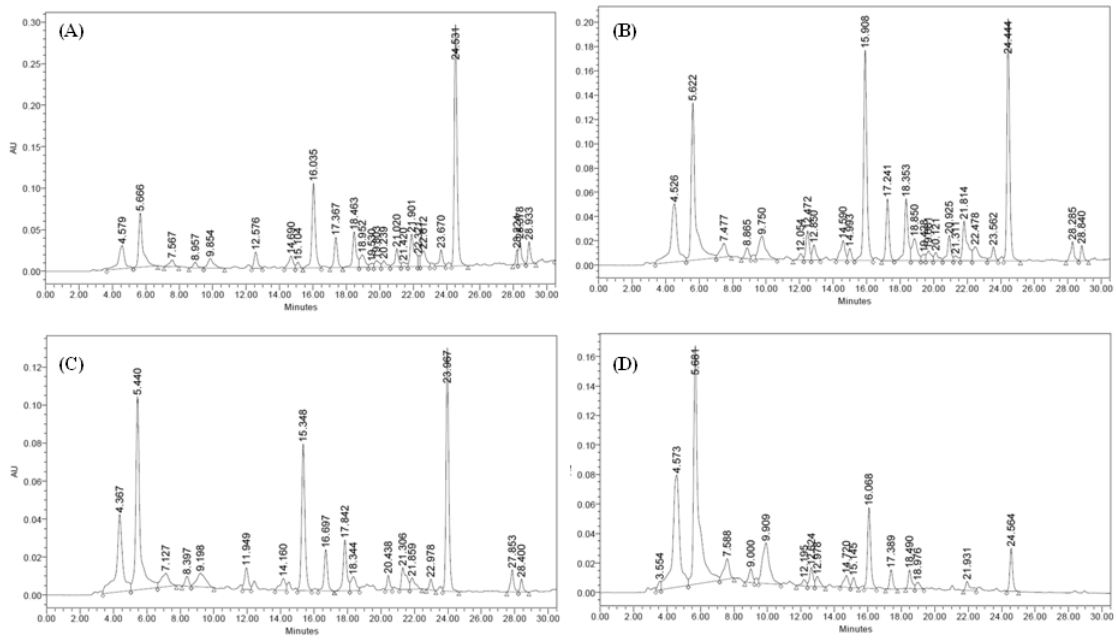

Fig. 1. Chromatographic profiles of propolis extracts obtained by different extraction conditions. (A) extract A1; (B) extract B1; (C) extract C3; (D) extract D1.

The substances shown in the chromatogram with smaller retention times, between two and ten minutes, are among the group of substances called phenolic acids, which are chlorogenic acids, cinnamic, *p*-coumaric acid, benzoic acid and its derivatives such as esters, ethers, among others (Hayacibara et al., 2005; Hu, Hepburn, Li, Chen, Radloff & Daya, 2005). Due to their chemical characteristics (small structures, containing alternating double bonds, solubility very close to the flavonoids and also often provides a phenyl grouping) this group exhibits good absorptivity in the ultraviolet, and therefore, good detection, which allows the quantification by HPLC. This similarity in the intensity of light absorption has caused many mixed results regarding the content of flavonoids, which justifies the need for the retention times of phenolic compounds present in propolis extracts.

3.2. Identification of phenolic acids in the propolis extracts

Tables 1 and 2 show the retention times obtained for *p*-coumaric acid, CAPE and Artepillin C, found in Brazilian propolis. The addition of the standard to the samples provides the detection and shows the variation in retention times between the profile of the pure substance and the substance associated with the analyte.

Table 1

Retention times obtained for the phenolic acids *p*-coumaric and CAPE from propolis extracts, standards and standards + propolis extracts

Extract[a]	Retention time (minutes)		
	Propolis extract	*p*-coumaric acid	Propolis extract + *p*-coumaric acid
A1	5.647	5.648	5.445
A2	5.591	5.648	5.587
A3	5.497	5.648	5.585
B1	5.646	5.648	4.968
B2	5.647	5.648	5.455
B3	5.547	5.648	5.459
C1	5.562	5.648	4.938
C2	5.443	5.648	5.434
C3	5.443	5.648	5.422
D1	5.653	5.648	5.400
D2	5.490	5.648	5.376
D3	5.488	5.648	5.355
	Propolis extract	CAPE	Propolis extract + CAPE
A1	18.449	17.972	17.621
A2	18.085	17.972	17.993
A3	17.907	17.972	17.902
B1	18.305	17.972	17.860
B2	18.343	17.972	17.855
B3	17.987	17.972	17.862
C1	17.981	17.972	17.667
C2	17.861	17.972	17.687
C3	17.867	17.972	17.700
D1	18.165	17.972	17.602
D2	17.992	17.972	17.598
D3	17.980	17.972	17.623

[a] Concentrations of propolis (A, B, C, D) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1, 2, 3) between 60.0 and 93.8% (v/v) of alcohol.

It is observed that the substances show small variation, which is due to the interaction between them (Fig. 1). When there is difference in the extraction process,

especially changes in the composition of the extraction liquid, other substances can be extracted, promoting significant differences in the retention times.

According to Table 1, it appears that both *p*-coumaric acid and CAPE were identified in the twelve propolis extracts. The *p*-coumaric acid, widely distributed in cereals, fruits and vegetables acts as an intermediary in the synthesis of many phenols (Clifford, 2000). Studies have shown that *p*-coumaric acid has antioxidant and antiinflammatory properties (Guglielmi, Luceri, Guglielmi, Lodovici, Giannini, Messerini & Dolara, 2003) and CAPE was identified as a major biological active compound of propolis, with chemopreventive and antitumor properties (Lee, Song, Mata-Greenwood, Kelloff, Steele & Pezzuto, 1999).

Table 2

Retention times obtained for Artepillin C from propolis extracts, standards and standards + propolis extracts

Extract[a]	Retention time (minutes)		
	Propolis extract	Artepillin C	Propolis extract + Artepillin C
A1	24.523	24.235	23.929
A2	24.491	24.235	24.205
A3	24.011	24.235	24.492
B1	24.393	24.235	23.925
B2	24.411	24.235	24.035
B3	24.051	24.235	24.042
C1	24.072	24.235	24.014
C2	23.969	24.235	24.005
C3	23.967	24.235	24.013
D1	24.374	24.235	24.023
D2	24.121	24.235	24.408
D3	24.122	24.235	24.247

[a] Concentrations of propolis (A, B, C, D) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1, 2, 3) between 60.0 and 93.8% (v/v) of alcohol.

Russo et al. (2002) investigated the antioxidant activity of a propolis extract with and without CAPE and found that the propolis extract containing CAPE had higher antioxidant activity than the extract without CAPE. The authors also found that CAPE,

singly, exhibited a strong antioxidant activity. Another substance that stands out in the group of phenolic acids present in the propolis is Artepillin C. Table 2 shows the retention times for Artepillin C, found in the propolis extracts.

The Artepillin C was found in all the propolis extracts, and Fig. 2 illustrates the presence of this substance in one of the propolis extracts.

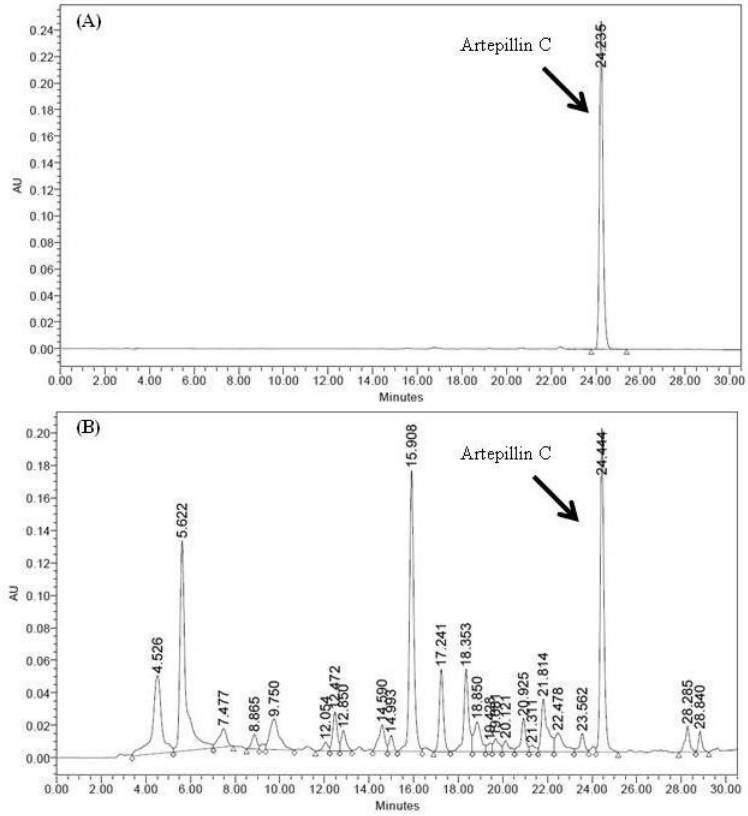

Fig. 2. Chromatograms of Artepillin C (A) and propolis extract B1 (B) obtained through the same chromatographic conditions.

Hayashi, Komura, Asaji, Ohishi & Yagi (1999) reported that the Artepillin C is the most abundant compound isolated in Brazilian propolis. Recently, several biological properties have been attributed to Artepillin C, such as antimicrobial, antioxidant and antitumor (Estrada et al, 2008). These findings reinforce the importance of studying the biological properties of propolis and its applications, since the Artepillin C is one of the major components of Brazilian propolis.

Regarding other phenolic acids, the gallic acid was found only in the extract D2, while chlorogenic acid was identified in extracts C1 and D1. The quantification of flavonoids and phenolic acids by HPLC carried out for the twelve propolis extracts

showed that the extracts D2, A3 and C1, in order, had higher extraction of total phenolic acids.

For benzoic acid, it is observed that this compound was not found in the extracts A1 and A3, however was found in ten other extracts, whereas caffeic acid was found in all the twelve extracts studied. The absence of chlorogenic acid, acid gallic and benzoic acid in some of the propolis extracts shows that different concentrations of propolis and alcohol levels can not only interfere with the amount of extracted substances, but also the extraction of certain compounds.

The phenolic acids are characterized by having a benzene ring, a carboxyl group and one or more hydroxyl and/or methoxy group in the molecule, which confers antioxidant properties to the plant. Also, they can also bind to each other or with other compounds. The most important combination of these acids occurs with caffeic acid, which associated with quinic acid, originates the chlorogenic acid. Several studies involving chlorogenic acid showed that this phenolic acid has anti-hyperglycemic and antioxidant activities (Chun, Kim & Lee, 2003). Another widely studied phenolic acid is the caffeic acid; there are numerous studies in the literature describing the antioxidant properties of this compound. Nardini, D'Aquino, Tomassi, Gentili, DiFelice & Scaccini (1995) showed that the caffeic acid inhibited, in a dose-dependent, the lipid peroxidation induced by cupric ions.

3.3. Identification of flavonoids in the propolis extracts

The retention times obtained for the flavonoids apigenin and chrysin are shown in Table 3. The flavonoid apigenin was identified in all the twelve extracts. Apigenin possesses diverse pharmacological effects including antioxidant and anti-carcinogenic potential (Silvan, Manoharan, Baskaran & Singh, 2010). The chrysin was also detected in all propolis extracts. Chrysin is an important constituent of propolis with several proven biological properties, such as antioxidant and anti-cancer (Khoo, Chua & Balaram, 2010).

Regarding other flavonoids, acacetin was not found only in the extract D1. This possibly occurred due to the alcohol level is insufficient (1 = lowest alcohol level) for the extraction of this flavonoid in higher concentrations of propolis (D = highest concentration of propolis). Naringenin was detected only in two extracts (C1 and D1).

Probably, naringenin is better extracted at lower alcohol level (1) and higher concentrations of propolis (C and D).

Table 3

Retention times obtained for apigenin and chrysin from propolis extracts, standards and standards + propolis extracts

Extract[a]	Retention time (minutes)		
	Propolis extract	Apigenin	Propolis extract + Apigenin
A1	17.404	17.642	16.389
A2	17.069	17.642	16.454
A3	16.818	17.642	16.733
B1	17.267	17.642	16.624
B2	17.208	17.642	16.957
B3	16.990	17.642	16.742
C1	17.058	17.642	16.713
C2	16.693	17.642	17.001
C3	16.695	17.642	17.189
D1	17.377	17.642	16.572
D2	16.875	17.642	16.602
D3	16.857	17.642	16.589
	Propolis extract	Chrysin	Propolis extract + chrysin
A1	21.020	21.217	20.052
A2	20.877	21.217	20.370
A3	20.532	21.217	20.525
B1	20.879	21.217	20.249
B2	20.924	21.217	21.352
B3	20.574	21.217	21.372
C1	20.745	21.217	20.427
C2	20.438	21.217	20.448
C3	20.448	21.217	20.452
D1	20.997	21.217	20.816
D2	20.591	21.217	20.355
D3	20.577	21.217	20.364

[a] Concentrations of propolis (A, B, C, D) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1, 2, 3) between 60.0 and 93.8% (v/v) of alcohol.

The flavonoid pinocembrin was detected in the twelve propolis extracts; however, galangin was not detected in four of these extracts (C3, D1, D2 and D3).

Galangin was not found in the three extracts with higher concentrations of propolis (D1, D2 and D3). It can be argued, therefore, that the extraction of this flavonoid is impaired in extracts with high concentrations of propolis. In the extract C3, galangin was not found, and this may be due to the interaction between the higher concentration of propolis and higher alcohol level which, probably, was not favorable to its extraction. Laskar, Sk, Roy & Begum (2010) isolated pinocembrin and galangin from the active fraction of an ethanolic propolis extract and found that the two flavonoids showed high 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, confirming the antioxidant potential of these two compounds.

3.4. Phenolic acids and flavonoids detected in the propolis extracts

Regarding the phenolic acids, four compounds were identified in the twelve extracts (caffeic acid, *p*-coumaric acid, CAPE and Artepillin C), while some compounds were detected only in some extracts (gallic acid - B1 and chlorogenic acid - C1 and D1). Benzoic acid was not found in the extracts A1 and A3. For flavonoids, apigenin, pinocembrin, and chrysin were detected in all extracts; however, naringenin has been identified only in the extracts C1 and D1. The flavonoids galangin and acacetin were detected in most extracts, except for C3, D1, D2 and D3 for galangin, and LLOSD1 for acacetin.

This result shows the importance of using the phenolic acids (caffeic acid, *p*-coumaric acid, CAPE and Artepillin C) and flavonoids (apigenin, chrysin and pinocembrin), as markers to quantify propolis and its derivative products.

Among all the compounds already identified in the propolis, Artepillin C is what attracts the attention of researchers, due to its many biological properties. Because it contains high levels of Artepillin C, the Brazilian propolis has been widely studied by researchers worldwide, especially the “green propolis”, produced from *Baccharis dracunculifolia*, considered the main source of Artepillin C. Chang et al. (2008) analyzed ethanolic extracts of green propolis and found a large amount of Artepillin C (63% of the base peak). The authors also observed the absence of chlorogenic acid in the extracts, which consisted mainly of cinnamic acid and derivatives (*p*-coumaric acid

and caffeic acid), flavonoids (acacetin), benzoic acid and some benzoates. Likewise, Chikaraishi et al. (2010) found that the main compounds present in an aqueous extract of Brazilian green propolis were the caffeoylquinic acid derivates (chlorogenic acid) and cinnamic acid derivatives (*p*-coumaric acid, drupanin, baccharin and Artepillin C).

Based on the results obtained from this study and in the literature, the propolis used in this work can be considered as green propolis, because, among the phenolic compounds identified, Artepillin C constitutes the main representative. Also, *Baccharis dracunculifolia* was present in the apiary where propolis was collected.

Fig. 4 illustrates the major phenolic compounds identified in propolis extracts.

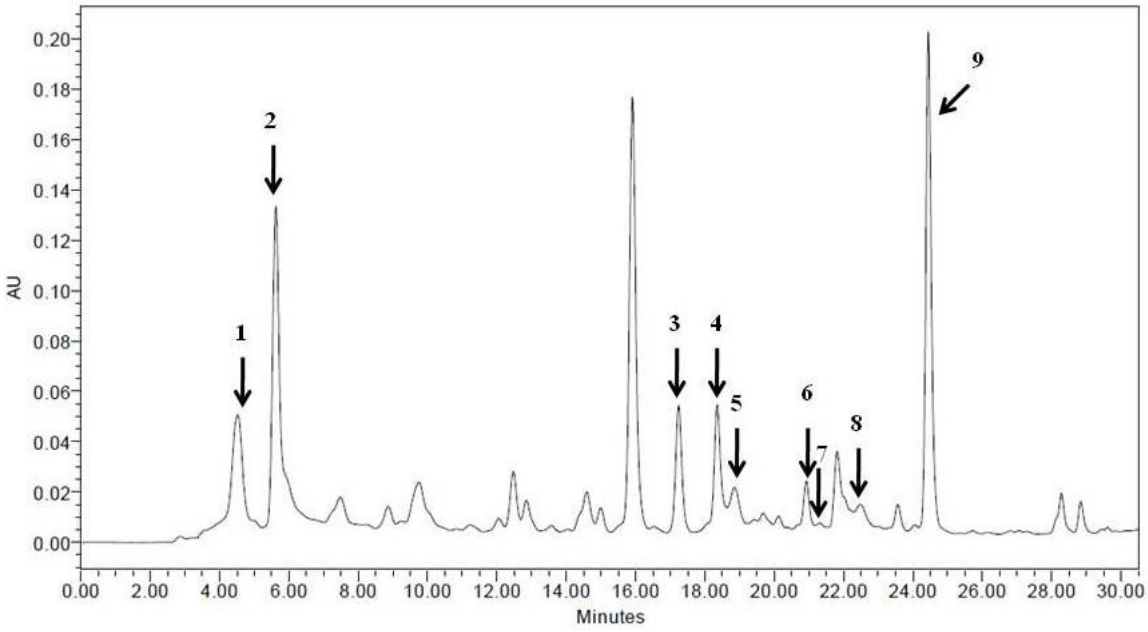

Fig. 3. Major compounds identified in the propolis extracts: caffeic acid (1), *p*-coumaric acid (2), apigenin (3), CAPE (4), pinocembrin (5), chrysin (6), galangin (7), acacetin (8) and Artepillin C (9).

In the propolis extracts were detected, in total, nine compounds. However, other compounds were detected (gallic acid, chlorogenic acid, benzoic acid and naringenin), but their extractions were only possible with specific propolis concentrations and alcohol levels. For benzoic acid, the reading range occurred at 284 nm rather than 310 nm and, despite being present in many of the extracts, its concentration is very small.

Finally, the determination of propolis plant sources is also important in increasing propolis production and attaining higher degrees of standardization. Unfortunately, due to the variation in the chemical composition of propolis,

standardization is likely to remain a constraint in apiculture for a long time to come (Salatino, Fernandes-Silva, Righi & Salatino, 2011). However, based on the results obtained from this work, it can be verified that, depending on the concentration of propolis and alcohol level used for the extraction, different phenolic compounds are extracted from propolis, resulting in extracts with different biological properties.

4. Conclusions

Seven phenolic acids and six flavonoids were identified in the propolis extracts, however, some of these phenolic compounds were found only in some specific extracts. It can be concluded, therefore, that the different concentrations of propolis and alcohol levels directly influence the extraction of some phenolic compounds, which alters the chemical composition of the propolis extracts, reinforcing the need of reliable criteria for chemical standardization of different propolis types.

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CAPÍTULO IV

(Normas: Revista Brasileira de Zootecnia)

Digestibility, Ruminal Parameters and Efficiency of Microbial Protein Synthesis of Dairy Cows Fed Diets containing Propolis-Based Products

Abtsract - Propolis-based products (LLOS), with different concentrations of propolis (B and C) and alcohol levels (1 and 3), were added in dairy cows' diets, in order to estimate the feed intake, total, (TD), ruminal (RD) and intestinal (ID) digestibility, pH and ruminal ammonia-nitrogen production (NH_3-N), rumen microbial synthesis and blood parameters. Four Holstein cows, weighing 550 ± 34.16 kg of BW and cannulated in the rumen were used, and distributed in a 4 x 4 Latin Square. The diet contained 59.19% of corn silage and 40.81% of concentrate, differing with the addition or not of LLOS: control (no LLOS), LLOS B1, LLOS C1 and LLOS C3. The ruminal pH, efficiency of microbial protein synthesis and blood parameters were not affected (P> 0.05) by the addition of LLOS, but there was an effect of LLOS (P <0.05) on feed intake, TD, RD, ID and NH_3-N production. The product LLOS B1 resulted in higher TD for DM (0.717 *vs*. 0.685), OM (0.737 *vs*. 0.703), CP (0.760 *vs*. 0.739), NDF (0.622 *vs*. 0.558) and TDN (0.747 vs. 0.712) only when compared to LLOS C3. The LLOS provided lower (P <0.05) RD of CP, the LLOS C1 reduced the ruminal NH_3-N production and the LLOS B1 increased the ID of CP compared to control diet. The addition of LLOS products interfere with ruminal metabolism and the product LLOS C1 had a positive effect on nitrogen metabolism, because it reduced the losses of N in NH_3 form, in the rumen. The different concentrations of propolis and alcohol levels used in the extracts preparation affected the extraction of the active compounds of propolis, since the products tested here showed different effects on the parameters evaluated and may be promising additives in dairy cows' diets, for example, LLOS B1 and C1.

Keywords: additive, flavonoids, phenolic acids, propolis extracts, soybean oil

Introduction

In the current livestock, is extremely important in the production process the aspects of the consumer market, the environment and animal welfare; and the use of nutritional additives in ruminant diets has been a practice necessary for this process becomes more efficient. The additives have a role as modulators of the end products of rumen fermentation, in order to increase protein and volatile fatty acids production, as well as reduce the methane (CH_4), carbon dioxide (CO_2) and ammonia (NH_3) production. As an alternative to the antibiotic additives (monensin, lasalocid) used in ruminant feed, but with its use prohibited in some countries such as the constituents of the European Union, many investigations have been performed with natural additives, such as plant extracts and, more recently, propolis extracts.

Propolis, a resinous material collected by worker bees from tree buds and secretions is known, mainly, for its antimicrobial, antioxidant, anti-inflammatory and anticarcinogenic properties. (Park et al., 2002; Kumazawa et al., 2004; Santos Neto et al., 2009). In studies with ruminants, propolis was effective in inhibiting the *in vitro* gas production by rumen microorganisms and allowed an increase in the specific digestion rate for carbohydrates (Stradiotti Junior et al. 2004a); also, reduced the NH_3 production *in vitro* and the specific activity of NH_3 production (Stradiotti Júnior et al., 2004b; Oliveira et al. 2006; Ozturk et al., 2010). Another effect of the use of propolis was the increase in dry matter (DM) and nutrients digestibility in buffalo (Prado et al., 2010a) and increase in the protein flow to the intestines in cattle (Prado et al. , 2010b).

Flavonoids are considered the main biologically active phenolic compounds present in propolis, although other compounds are also involved, such as cinnamic acid derivates and its esters and diterpenes (Castro et al., 2007). Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) is one of the major phenolic acids found in Brazilian propolis and has multiple biological activities, among them antimicrobial, antioxidant and antitumor (Shimizu et al., 2004; Estrada et al., 2008).

Propolis, a natural additive, may be an alternative to the antibiotics commonly used in cattle feed. However, to be officially accepted by major health agencies, propolis needs a chemical standardization to ensure its quality, safety and efficacy (Bankova, 2005), since its composition is closely related to the ecology of the flora of each region visited by bees (Park et al., 2002), which directly influence their active

compounds and, therefore, their effects on ruminal microbiota. Still, the concentration of propolis and alcohol level used for the extraction of the active substances can interfere with the chemical composition and antioxidant activity of the extracts (Cottica et al., 2011). Therefore, the objective was to evaluate the effects of three propolis-based products, with different concentration of propolis and alcohol level, on feed intake, partial and total digestibility, ruminal and blood parameters and microbial efficiency in lactating dairy cows.

Material and Methods

The experiment was carried out in the city of Maringá, Paraná state, Brazil. Four primiparous Holstein cows were used, with 147 days of lactation, weighing 550 ± 34.16 kg of body weight (BW), cannulated in the rumen, housed in individual cages and subjected to two daily milkings (6h and 15h). The animals were randomly assigned to a 4 x 4 Latin square, with four periods and four treatments. The propolis-based products differed in the concentration of propolis (B and C, between 5.0 and 30.0% (w/v)) and water-alcohol solutions (1 and 3, between 60.0 and 93.8% (v/v)), prepared according to the methodology developed by Franco & Bueno (1999). The propolis samples were obtained from the apiary of the Experimental Farm of Iguatemi (FEI), belonging to the Universidade Estadual de Maringá, Paraná State, Brazil, being certified as organic. The apiary is located within a reserve of eucalyptus (*Eucalyptus* sp.) surrounded by native forest, with the presence of alecrim-do-campo (*Baccharis dracunculifolia*).

Propolis-based product (LLOS), a powder, contains dried propolis extract and is registered in the National Institute of Industrial Property – Brazil, under no. 0605768-3. The preparation of LLOS consists of the hydroalcoholic extraction of raw propolis to release its active substances – flavonoids, mainly. Subsequently, the alcohol is evaporated with the aid of a rotary evaporator and the extract is dried. Due the amount of extract supplied to the animals is too small, was added to the extract an excipient (corn and soybean meal, 50:50) to add volume facilitating the animal feeding. The daily amount of some flavonoids and phenolic acids provided to the animals (through the LLOS products) is shown in Table 1. The quantification of these compounds was performed using high-performance liquid chromatography (HPLC).

Table 1 – Composition in flavonoids and phenolic acids identified in the propolis-based products (LLOS)[1] supplied daily for dairy cows

	LLOS B1	LLOS C1	LLOS C3
	mg/g of dry extract		
Chlorogenic acid	n.d.	0.24	nd
Caffeic acid	4.06	4.75	3.10
p-coumaric acid	7.16	8.15	5.27
Benzoic acid	0.59	1.20	0.45
CAPE	2.73	2.68	1.49
Artepillin C	7.59	7.27	4.62
Apigenin	7.66	5.69	3.72
Pinocembrin	4.92	3.62	2.33
Galangin	1.49	n.d.	n.d.
Chrysin	3.90	2.65	1.61
Acacetin	4.06	3.65	2.04

nd = not detected. [1]Concentrations of propolis (B and C) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1and 3) between 60.0 and 93.8% (v/v) of alcohol.

The experimental diets (Table 2) containing, 59.19% of corn silage and 40.81% of concentrate, differed with the inclusion or not of the LLOS products constituting, therefore, in four treatments: control (no additive), LLOS B1, LLOS C1 and LLOS C3.

Table 2 – Chemical composition and proportion of ingredients used in the experimental diet

	g/kg[1]									
	DM	OM	CP	EE	NDF	ADF	TC	NFC	TDN	Diet (%)
Corn silage	292.7	962.3	72.7	30.3	606.7	337.1	856.2	249.6	634.4	59.19
Soybean meal	898.0	935.1	462.1	14.9	182.3	100.4	433.2	250.9	806.8	19.77
Ground corn	878.7	985.1	91.2	18.3	165.2	37.6	869.0	704.0	832.0	5.26
Wheat meal	857.1	948.1	170.7	23.2	458.7	148.8	754.2	295.5	715.4	10.48
Soybean oil	995.7	997.0	-	991.3	-	-	-	-	2139	2.86
V.M. suppl.[2]	990.0	-	-	-	-	-	-	-	-	1.98
Limestone	991.4	-	-	-	-	-	-	-	-	0.32
Am.sulfate	990.0	-	1250	-	-	-	-	-	-	0.14
Exp. diet	539.4	934.1	160.6	52.60	451.8	236.9	722.4	270.6	714.9	100.0

[1]DM= dry matter, OM= organic matter, CP= crude protein, EE= ether extract, NDF= neutral detergent fiber, ADF= acid detergent fiber, TC= total carbohydrates, NFC= non-fiber carbohydrates, TDN= total digestible nutrients. [2]Composition of vitamin and mineral supplement (per kg of product): 146 g of calcium, 51 g of phosphorus, 20 g of sulfur, 33 g of magnesium, 93 g of sodium, 28 g of potassium, 30 mg of cobalt, 400 mg of copper, 10 mg of chromium, 2.000 mg of iron, 40 mg of iodine, 1.350 mg of manganese, 15 mg of selenium, 510 mg of fluoride, 1.700 mg of zinc, 135.000 IU of Vit. A, 78.000 IU of Vit D3 and 450 IU of Vit. E.

The chemical composition of the experimental diet is presented in Table 2, which was formulated according the recommendations proposed by NRC (2001) for lactating cows with approximately 550 kg BW, 21 weeks of lactation and with estimated milk production of 25.0 kg, with 3.8% fat. The net energy for lactation (NEL) was estimated using the equation: NEL (Mcal/kg) = 0.0245 x %TDN – 0.12 (NRC, 2001), obtaining the value of 1.63 Mcal/kg.

The propolis-based products were placed into the rumen via ruminal cannula at the time of feeding. The animals received two daily doses of LLOS (7.5 g) previously weighed in hygroscopic paper, with a total of 15.0 g of LLOS/day. The ration, weighed daily, was provided *ad libitum*, so that the refusals represented 10% of the total. The cows were weighed at the beginning and at the end of each collection period, in order to estimate supplied DM according to the BW.

Daily feed intake was estimated by the difference between the supplied feed and refusals in the trough. During the experimental period, samples of the supplied feed and refusals were collected and a representative composite sample was drafted per animal in each treatment. The samples were stored in plastic bags properly identified and stored at a freezing temperature (-10°C) for further analysis.

The experiment consisted of four experimental periods of 21 days each, where 14 days were for adaptation and seven days for collection. From the second to fifth day of the collection period feces and omasal digesta were sampled. Fecal samples (100 g) were taken directly from the rectum and the omasal digesta samples (400 mL) were collected by suction of the omasum content, according to the technique described by Leão et al. (2005). On the first day, the collection was performed at 20 h, the second day at 16 h, the third day at 12 h and on the fourth day at 8 h, totaling four samples (feces and digesta) per animal in each period.

After the collection period, feed, feces and digesta samples were dried in a ventilated oven (55°C for 72h) and ground to 1 mm, and then were mixed in equal quantities, based on the dry weight, to form composite samples.

In the last two days of each experimental period, ruminal fluid were collected via cannula to determine the pH and ammonia nitrogen (NH_3-N), and urine was collected to determine the efficiency of microbial synthesis. The collection started before the first feeding (8h), which was taken as time zero (0), 2, 4, 6 and 8 hours post feed, with five samples/animal/period. For NH_3-N determination, the material was

filtered to obtain 50 mL of ruminal fluid. Immediately after collection, it was determined the pH of the sample through a digital pH meter and then added 1 mL of sulfuric acid (H_2SO_4) 1:1. To determine the daily flows of DM and digesta, the chromic oxide (Cr_2O_3) was used as external marker. Two intra-ruminal doses (5.0 g) were provided daily (at 8h and 16h), previously weighed in hygroscopic paper, for a total of 10.0 g Cr_2O_3/day. The NH_3-N determination was performed by distillation with potassium hydroxide 2 N, according to the method described by Vieira (1980).

The partial and total digestibility coefficients for DM and nutrients were estimated according to the equations described by Coelho da Silva & Leão (1979). The analysis to determine dry matter (DM, method no. 934.01), organic matter determined by ash (OM, method no. 924.05), crude protein (CP, method no. 920.87) and ether extract (EE, method no. 920.85) in the samples milled to 1 mm, were conducted in accordance to the AOAC (1990). Neutral detergent fiber (NDF) was determined according to Van Soest et al. (1991) and acid detergent fiber (ADF) determined according to method no. 973.18 (AOAC, 1990). The total carbohydrates (TC) were obtained by using the following equation: TC = 100 - (% CP +% EE +% Ash), (Sniffen et al., 1992). Non-fiber carbohydrates (NFC) were determined by the difference between TC and NDF (without correction for protein). TDN content of the experimental diets was calculated using the following equation: %TDN = %DCP + %DNDF + %DNFC + %(DEE x 2.25), where: DCP = digestible crude protein, DNDF: digestible neutral detergent fiber, DNFC = digestible non-fiber carbohydrates, DEE = ether extract.

In order to determine microbial production, spot urine samples were collected approximately four hours after feeding, during voluntary urination. The samples were filtered to prevent possible contamination. An aliquot of 10 mL of urine was diluted in 40 mL of sulfuric acid (H_2SO_4) 0.036 N, in order to avoid bacterial destruction of purine derivatives and uric acid precipitation. Urine samples were stored under refrigeration (5°C) and subsequently analyzed for concentrations of creatinine, allantoin, uric acid and urea. On the same day, samples of milk were collected from the first and second milking, and then a composite sample was made for further analysis. A 10 mL aliquot of milk was mixed with 5 mL of trichloroacetic acid ($C_2HCl_3O_2$) at 25%, filtered and stored at 5°C, for subsequent analysis of urea and allantoin.

The analyses of allantoin were performed using the methods described by Chen & Gomes (1992). For the determination of creatinine, uric acid and urea, analyzes were performed using commercial kits (Labtest). From the concentration of creatinine in the spot urine sample, urinary volume was estimated (L), dividing the daily excretion of creatinine (mg/kg BW) by the creatinine concentration (mg/L). For determination of daily creatinine excretion per kg of BW, the average value of 23.41 mg/kg of BW was used, obtained by Oliveira et al. (2001), which determined the creatinine excretion of Holstein cows fed diets composed of 60:40 forage-to-concentrate, characteristics similar to this study. The microbial nitrogen (N) production was calculated from the amount of absorbed purines, which was estimated from the sum of the excretion of purine derivatives (PD) in milk and urine, and the synthesis of microbial N compounds in the rumen was calculated based on the absorbed purine, according to Chen & Gomes (1992). The estimate of microbial protein (MP) was obtained by multiplying the microbial N synthesis by 6.25, while the efficiency of microbial protein synthesis (EMPS) was determined as: EMPS (g/kg) = MP (g)/TDNI (kg), where TDNI = total digestible nutrients intake.

For the determination of blood urea, blood samples were collected on the 21^{o} day of each experimental period, four hours after the supply of the first feeding. Immediately after sampling, the tubes (containing heparin) were centrifuged at 2500 rpm for 15 minutes to separate the plasma and serum. The centrifuged samples were transferred to plastic tubes properly identified, stored in a thermal box and taken immediately to the laboratory to perform the analysis, using a commercial kit in an automatic analyzer for blood biochemistry (Merck Vitalab Selectra®).

Data were interpreted by analysis of variance using the GLM procedure of SAS statistical software (2001). The mathematical model used for the analysis was: $Y_{ijk} = \mu + A_i + P_j + T_k + e_{ijk}$, where: Y_{ijk} = observed variables, μ = overall mean, A_i = effect of animal i, ranging from 1 to 4; P_j = effect of the period j varying from 1 to 4; T_k = k effect of the treatment, ranging from 1 to 4; e_{ijk} = random error. Statistical analyzes of ruminal parameters (pH, and NH_3-N) were performed in a split-plot design, with treatments in the plots and collection time as subplots. Differences between treatment means were determined by Tukey test. Tests that had $\alpha = 0.05$ were considered statistically significant; those that had values $\alpha = 0.10$ suggested trends.

Results and Discussion

The amount of flavonoids and phenolic acids supplied to the animals, based on DM intake, is presented in Table 3. It is verified that LLOS B1 has the highest content of flavonoids, whereas LLOS C1 has the highest content of phenolic acids. The LLOS C3 was the product that had the lowest concentration of phenolic compounds.

Table 3 – Total flavonoids quantified in apigenin and total phenolic acids quantified in Artepillin C in LLOS products supplied daily for dairy cows

Compounds	Propolis-based product[1]			P	CV[2]
	LLOS B1	LLOS C1	LLOS C3		
Total flavonoids	2.81a	2.14b	1.22c	<0.001	2.72
Artepillin C and CAPE	0.40a	0.41a	0.25b	<0.001	3.40
Total phenolic acids[3]	1.00b	1.13a	0.71c	<0.001	3.68

[1]Concentrations of propolis (B and C) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1 and 3) between 60.0 and 93.8% (v/v) of alcohol. [2] Sum of the phenolic acids grouped at the beginning of the chromatogram with CAPE and Artepillin C.

The addition of LLOS influenced (P<0.05) the NDF intake and DM, CP and TC intakes (P<0.10) (Table 4). The intake of NDF was higher (P<0.01) for the diet with the addition of LLOS B1 than the others diets, probably due to the higher fiber digestibility observed.

The effects on feed intake of diets containing propolis-based products does not seem to affect the feed intake when propolis is supplied in the powder form, as reported in the literature (Valero, 2009; Simioni, 2011; Zawadzki et al., 2011; Aguiar et al., in 2012), in feedlot cattle diets and forage-based diets for cattle (Prado et al., 2010b) and buffalo (Prado et al., 2010a). Similarly, Stelzer et al. (2009) tested two levels of concentrate (20% and 40% of DM) and ethanolic propolis extract (30% w/v) for dairy cows and found no changes in feed intake. However, Loureiro et al. (2007) found a reduction in DM intake (DMI) in lambs fed diets containing 15 mg and 30 mg of propolis extract/kg BW when compared to control. For animals receiving control diet, 15 mg and 30 mg of propolis, the values found for CMS were 0.36, 0.28 and 0.23 kg/day, respectively. Probably, the forms of inclusion of propolis in the diet (powder, liquid or directly into the rumen) and dosages, together with the type of diet and animal, are responsible for the results obtained for feed intake so far.

Table 4 – Feed intake, ruminal[1] (RD) and intestinal[2] (ID) digestibility of DM and nutrients of diets with or without (CON) the addition of propolis-based products (LLOS)

Parameters	Diets[3]				CV[4]	P
	CON	LLOS B1	LLOS C1	LLOS C3		
			Dry matter			
Intake (kg/day)	15.66ab	16.34a	15.60ab	15.31b	2.70	0.064
Omasal flow (kg/day)	7.62	8.11	8.04	7.44	4.57	>0.100
RD (kg/kg)	0.513a	0.503ab	0.483b	0.514a	2.12	0.022
Fecal flow (kg/day)	4.87	4.61	4.64	4.82	6.35	0.197
ID (kg/kg)	0.362b	0.430a	0.422a	0.352b	5.71	0.004
			Organic matter			
Intake (kg/day)	14.60ab	15.23a	14.55ab	14.28b	2.65	0.062
Omasal flow (kg/day)	6.46ab	6.84a	6.80ab	6.26b	4.12	0.063
RD (kg/kg)	0.557a	0.550a	0.532b	0.561a	1.63	0.013
Fecal flow (kg/day)	4.25	4.00	4.05	4.23	6.54	0.125
ID (kg/kg)	0.343b	0.414a	0.404a	0.323b	7.51	0.008
			Crude protein			
Intake (kg/day)	2.51ab	2.67a	2.52ab	2.46b	3.75	0.079
Omasal flow (kg/day)	1.87b	2.16a	2.05ab	1.99ab	5.52	0.048
RD (kg/kg)	0.256a	0.189b	0.182b	0.190b	10.23	0.007
Fecal flow (kg/day)	0.63	0.64	0.69	0.64	5.94	0.214
ID (kg/kg)	0.657b	0.700a	0.663b	0.678ab	2.33	0.030
			Neutral detergent fiber			
Intake (kg/day)	6.84b	7.15a	6.81b	6.61b	2.07	0.009
Omasal flow (kg/day)	2.97ab	3.20a	3.23a	2.88b	4.67	0.031
RD (kg/kg)	0.565	0.552	0.525	0.563	3.88	0.113
Fecal flow (kg/day)	2.92	2.70	2.74	2.92	7.56	0.375
ID (kg/kg)	0.018ab	0.158a	0.152a	-0.023b	103.45	0.037
			Total carbohydrates			
Intake (kg/day)	11.26ab	11.68a	11.19ab	11.00b	2.44	0.059
Omasal flow (kg/day)	4.05ab	4.37a	4.30a	3.87b	4.85	0.041
RD (kg/kg)	0.639a	0.625ab	0.615b	0.648a	1.82	0.027
Fecal flow (kg/day)	3.54	3.28	3.29	3.52	7.71	0.396
ID (kg/kg)	0.126b	0.247a	0.235a	0.089b	28.77	0.009

Different letters in the same line are statistically different (P<0.05, P<0.10) by Tukey test. [1]based on the amount ingested. [2]based on the amount that reached the duodenum. [3]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm. [4]Coefficient of variation.

There was significant effect (P<0.05) of the propolis-based products (LLOS) on ruminal digestibility (DR) of DM, OM, CP and CT (Table 4). The LLOS acted in the rumen and lower fermentation of DM and nutrients, especially for protein, was observed with LLOS B1 and C1. Similarly, Prado et al. (2010a) found that the smallest reduction in rumen fermentation in buffalo occurred for diets containing LLOS C1 and monensin, which did not differ among themselves, followed by LLOS B3.

The LLOS provided lower crude protein RD (CPRD) when compared to control (P<0.05), corroborating Prado et al. (2010a, b), which also reported lower CPRD for

LLOS (P<0.05) in cattle and buffaloes. For cattle, Simioni (2011) observed a lower CPRD for LLOS C1 (P<0.05) compared to monensin, however, did not differ from control. These data suggest that LLOS are positively acting in the N metabolism in the rumen, by reducing the NH_3-producing bacteria and, therefore, increasing the flow of microbial protein to the intestine, which was observed in this study, since the diets containing propolis increased the intestinal flow of CP (P <0.05) and, consequently, increased intestinal digestion of CP compared to control diet.

There was no effect (P>0.05) of LLOS on the neutral detergent fiber RD (NDFRD), however, there was an increase in the flow of NDF (P<0.05) to omasum with the diets containing LLOS B1 and C1, when compared to LLOS C3, but these did not differ from control. Prado et al. (2010a) found lower NDFRD (percentage of ingested) for monensin and LLOS C1 (P<0.05) in forage-based diets for buffaloes, when compared to control diet.

For TC, there was greater RD for LLOS C3 (P<0.05), which was similar to control, and lower total carbohydrates RD (TCRD) for LLOS B1 and C1, but the latter did not differ from control. This difference in TCRD between the LLOS affected the total digestibility (TD) of total carbohydrates, with higher TD for LLOS B1 and lower for LLOS C3.

Propolis-based products affected (P<0.05) the intestinal digestibility (ID) of DM, OM, NDF, CP and CT (Table 4). Diets LLOS B1 and C1 had higher dry matter intestinal digestibility (DMID) (P<0.05) compared to control and LLOS C3, with the same behavior observed for organic matter ID. These results are in agreement with Prado et al. (2010a), who found higher DM and OM intestinal digestibility (P<0.05) for diets containing additives (monensin, LLOS C1 and B3) compared to control, whereas monensin and LLOS C1 promoted greater DMID and LLOS C1 promoted higher OMID.

For crude protein, there was a greater intestinal digestion (P<0.05) for the product LLOS B1. This result is very favorable, because the ultimate goal of proper nutrition is to maximize rumen microbial growth and the amount of RDP that is captured by the rumen microbial cells. Maximizing the capture of N degradable not only improves the supply of amino acids into the small intestine, but also reduces the loss of N (Bach et al., 2005). The LLOS may be, thus, acting on the main ammonia-producing bacteria, such as *Prevotella ruminicola* and *Peptostreptococcus* sp., reducing

energy loss and increasing the flow of microbial protein to the intestine, where they will be absorbed.

There was higher NDF intestinal digestibility (ID) for diets containing LLOS B1 and C1, and the same happened for total carbohydrates ID. These data agree with those observed by Prado et al. (2010a), who found higher ID of DM, OM, NDF and CT for LLOS C1, monensin and LLOSB3. With the addition of additives, lower values in ruminal digestibility and changes in the primary site of digestion (rumen) were observed for most nutritional components, which reflected significant increases in intestinal digestibility.

There was effect of propolis-based products ($P<0.05$) on total digestibility (TD) of DM, OM, CP, NDF and TDN, with trends ($P<0.10$) to TC and ADF total digestibility (Table 5). The product LLOS B1 resulted in greater total digestibility of DM, OM, NDF, ADF and CT ($P<0.05$) compared to LLOS C3, but did not differ ($P>0.05$) from control and LLOS C1. Stelzer et al. (2009) observed no effect of propolis and propolis × concentrate level interaction on apparent digestibility of DM and nutrients in dairy cows. On the other hand, in buffaloes, Prado et al. (2010a) found higher total digestibility of DM, OM, NDF, TC and TDN with the addition of LLOS C1 when compared to control and monensin diets, but this did not occur with the addition of additives in cattle diets (Prado et al. 2010b). It is noteworthy that the dosage of the LLOS to the animals in the studies of Prado et al. (2010a,b) was three times lower than that used in this experiment. In this work, the greatest DM and OM total digestibility caused by the inclusion of the LLOS B1 compared to LLOS C3 can be attributed to the daily amounts of flavonoids (LLOS B1 = 2.81 ppm and LLOS C3 = 1.22 ppm) and phenolic acids (LLOS B1 = 1.00 ppm and LLOS C3 = 0.71 ppm) provided, since they are responsible for the biological activities of propolis, particularly antimicrobial.

New evidence involving the mechanisms of antimicrobial action of flavonoids have been proposed: inhibition of cell wall synthesis and cell membrane (Cushnie & Lamb, 2011). According to these authors, based on previous studies and on new evidence, a single flavonoid may have different mechanisms of action, and, in propolis, these can be influenced by the synergism between the other phenolic compounds, which difficult the understanding of its antimicrobial activity.

Table 5 – Dry matter and nutrients total digestibility (TD) and total digestible nutrients (TDN) of diets with or without (CON) the addition of propolis-based products (LLOS)

Parameters[1]	Diets[2] (kg/kg)				CV[3]	P
	CON	LLOS B1	LLOS C1	LLOS C3		
DMTD	0.689ab	0.717a	0.702ab	0.685b	1.75	0.034
OMTD	0.709ab	0.737a	0.721ab	0.703b	1.70	0.030
CPTD	0.745ab	0.760a	0.725b	0.739ab	1.44	0.022
EETD	0.912	0.920	0.916	0.908	1.13	0.385
NDFTD	0.573ab	0.622a	0.598ab	0.558b	3.77	0.025
ADFTD	0.552ab	0.591a	0.570ab	0.526b	4.64	0.054
TCTD	0.685ab	0.718a	0.706ab	0.680b	2.39	0.053
NFCTD	0.860	0.870	0.881	0.867	1.69	0.316
TDN	0.719ab	0.747a	0.732ab	0.712b	1.61	0.022

Different letters in the same line are statistically different (P<0.05, P<0.10) by Tukey test. [1]DM= dry matter, OM= organic matter, CP= crude protein, EE= ether extract, NDF= neutral detergent fiber, ADF= acid detergent fiber, TC= total carbohydrates, NFC= non-fiber carbohydrates. [2]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm. [3]Coefficient of variation.

Among the phenolic acids, we highlight the caffeic acid phenethyl ester (CAPE) and Artepillin C, both found in the LLOS (LLOS B1 = 0.40 ppm and LLOS C3 = 0.25 ppm) and with strong antimicrobial activity; however, their mechanisms of action has not been fully elucidated (Estrada et al., 2008; Bankova, 2009).

The greater CP total digestibility (P<0.05) obtained for LLOS B1 compared to LLOS C1 differ from previous work with the products LLOS, which found no significant effects of propolis on CPTD in cattle and buffaloes, testing different extracts and/or different dosages of LLOS (Prado et al., 2010b; Valero, 2010, Daniel, 2011; Simioni, 2011, Aguiar et al., 2012). However, effects on ruminal metabolism of CP were observed, as the increased of microbial protein flow to the intestine (Prado et al., 2010b).

Regarding lipids, the effect on EE total digestibility (TD) has been reported with the addition of propolis, although this study EETD was not affected (P>0.05) by adding the LLOS. Prado et al. (2010b) observed that the EETD in cattle was reduced (P <0.05) by inclusion of LLOS C1 and B3, compared to control diet and monensin, with more pronounced negative effect for the diet containing LLOS B3. However, these results contradict those found by Valero (2010), which showed higher EETD with LLOS C1 when compared to control diet and monensin, and Simioni (2011), who reported a tendency (P=0.08) of LLOS C1 to increase EETD, compared to the control diet. But, is important to note the forage-to-concentrate ratio supplied to the animals and the dosages

provided of LLOS, which were different between experiments. While Prado et al. (2010b) provided forage-based rations; Valero (2010) and Simioni (2011) used more concentrated diets in feedlot cattle. Generally, *Anaerovibrio lipolytica* would be expected to dominate ruminal lipase activity in animals receiving mainly concentrate feeds, but, because *A. lipolytica* lacks the ability to hydrolyse galacto- and phospholipids, other lipolytic species would be expected to predominate in grazing animals, such as *Butyrivibrio* spp., which hydrolyzes phospho- and galactolipids, but did not break down triacylglycerols, the main substrate of *A. lipolytica* (Lourenço et al., 2010). Therefore, it is possible that *Butyrivibrio* spp. is more sensitive to propolis, which may influence on lipid digestion.

The products LLOS influenced the digestion of dietary fiber fraction. The inclusion of the product LLOS B1 in the diet resulted in higher coefficients of NDF ($P<0.05$) and ADF ($P=0.054$) total digestibility compared to LLOS C3, but did not differ from control diet and LLOS C1. Similarly, others authors have reported the effect of propolis on fiber digestibility. Prado et al. (2010a) found that LLOS C1 promoted higher ($P <0.05$) NDF total digestibility, when compared to monensin.

The same behavior for LLOS C1 and monensin was observed for the ADF total digestibility. Importantly, the doses of the products LLOS used by Prado et al. (2010a) were lower than those used in this study. Aguiar et al. (2012) also observed an increase in ADF digestibility ($P=0.08$), when the dose of LLOS C1 provided was twice of that used by Prado et al. (2010a).

Thus, higher TC digestibility ($P=0.053$) was found for the inclusion of LLOS B1, compared to LLOS C3. According to Prado et al. (2010b), the observed differences between the products LLOS may be related not only to the concentration of flavonoids, but also to the alcohol levels used in the extraction of active substances of LLOS. For the same concentration of propolis in different alcoholic extractions, Prado et al. (2010c) concluded that in the higher alcohol content may be occurring solubilization resins and waxes in the propolis, which were influencing the release of active substances. This assumption can be supported by the results obtained in the quantification study, where LLOS C3 showed a lower concentration of flavonoids and phenolic acids than products LLOS B1 and C1 (the numbers 3 and 1 represent, respectively, the higher and lower alcohol levels used in the preparation of products).

There was no effect (P>0.05) for the interaction treatment x collection time after feeding and treatment for the values of pH in the rumen of dairy cows, however, there was effect of collection time (P<0.05) (Figure 1).

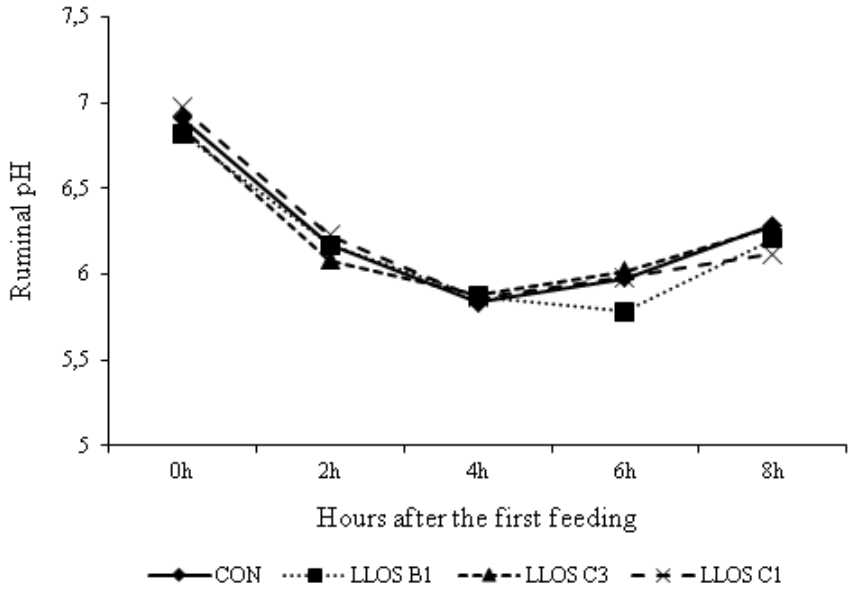

Figure 1 – pH of the rumen fluid of dairy cows in function of the time after feeding with the addition of propolis-based products (LLOS).

The ruminal pH according to the hours after feeding showed a quadratic (pH = 6.86944 – 0.42107X + 0.042898X^2, r^2 = 0,736%) with an estimated minimum of 5.83 at 4.9h. In previous work using LLOS in ruminant diets, there was no influence of propolis on ruminal pH (Prado et al., 2010a,b; Daniel, 2011). However, Simioni (2011) found that ruminal pH remained higher (P<0.05) in the diet containing the LLOS B1 (two doses) and LLOSC1 (three doses) compared to monensin, but did not differ from control diet.

There was no effect of the interaction treatment x collecting time (P>0.05) after feeding for NH_3-N concentration in rumen fluid (Figure 2). The behavior of NH_3-N in function of time after feeding was quadratic, where NH_3-N = 16.4810 + 7.96253x – 0.871208x^2, with r^2= 0,951%. The highest estimated concentration of NH_3-N was 34.67 mg/dL of ruminal fluid at 4.6 h after feeding and the minimum concentration was 16.48 mg/dL of ruminal fluid at 0h before feeding.

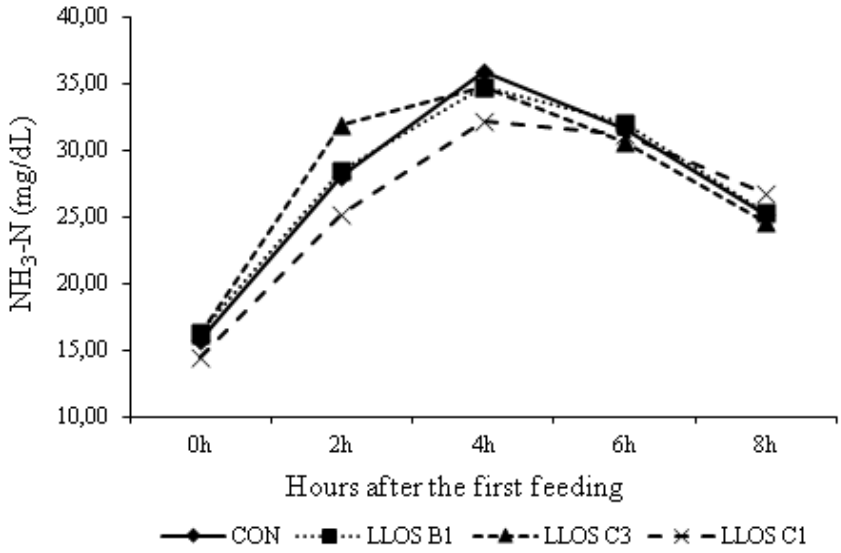

Figure 2 – NH_3-N concentration in the rumen fluid of dairy cows in function of the time after feeding with the addition of propolis-based products (LLOS).

The mean concentrations of NH_3-N in the rumen were influenced (P<0.05) by adding the products LLOS in the diet (Table 6). This behavior was not observed in previous studies with the products LLOS in the diets of cattle and buffaloes, which found no effects of propolis on NH_3-N concentrations (Prado et al., 2010a,b; Daniel, 2011; Simioni, 2011).

Table 6 – Ruminal pH and ammonia nitrogen (NH_3-N) of dairy cows fed diets with or without (CON) the addition of propolis-based products (LLOS)

Parameters	Diets[1]				P	CV[2]
	CON	LLOS B1	LLOS C1	LLOS C3		
pH	6.24	6.17	6.22	6.23	0.058	2.60
N-NH_3 (mg/dL)	27.27a	27.37a	25.94b	27.63a	0.0001	5.57

Different letters in the same line are statistically different (P<0.05, P<0.10) by Tukey test. [1]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm [2]Coefficient of variation.

However, propolis appears to reduce NH_3 production. Oliveira et al. (2004) studied the effect of monensin and propolis extract on NH_3 production and the *in vitro* degradability of different protein sources (trypticase, soybean meal and fish meal), with or without monensin and propolis, and found that monensin and propolis were both effective in inhibiting the NH_3 production to soybean meal and trypticase. In a later study, Oliveira et al. (2006) investigated the *in vitro* effects of monensin and propolis extract on ruminal fermentation of amino acids and found that propolis presented as

more effective than monensin in reducing the NH_3 production from cultures of rumen microorganisms in a medium containing casein hydrolyzed.

Similarly, Ozturk et al. (2010) investigated the effects of different concentrations of ethanolic propolis extracts on *in vitro* microbial fermentation and found that the concentration of NH_3-N in rumen fluid was reduced ($P<0.05$) using a dose-dependent mechanism, to 24% and 39% with the addition of low and high concentrations of the propolis extract, respectively.

Regarding protein degradation, it is important to emphasize the role of protozoa, since they showed sensitivity to LLOS (Ríspoli et al., 2009). The most important aspect of protozoa is their ability to engulf large molecules, proteins, carbohydrates and rumen bacteria (Van Soest, 1994). Because of the protozoa are not able to use NH_3-N (Onodera et al., 1977), a fraction of the engulfed insoluble protein returns to the ruminal fluid in the form of soluble protein (Dijkstra, 1994). This is one of the main reasons why the defaunation decreases the concentration of NH_3-N in the rumen (Eugene et al., 2004). Therefore, products LLOS (especially LLOS C1) can be not only acting on NH_3-producing bacteria, but also on protozoa.

The propolis-based products (LLOS) had no effect ($P>0.05$) in the excretion of purine derivatives in urine and milk, microbial protein synthesis (g/day) and microbial efficiency (g MPS/ kg of TDN) (Table 7).

Similarly, in previous studies with the products LLOS in ruminant diets, the microbial protein synthesis and efficiency of microbial protein synthesis was not affected (Valero, 2010, Daniel, 2011; Simioni, 2011, Aguiar et al., 2012).

For the excretion of allantoin as % of total purine excreted in urine, it was obtained an average of 90.06%. Accoridng to Chen & Gomes (1992), the excretion of allantoin ranges from 80-85% of the total amount excreted in the urine, a value lower than that found. However, Chizzotti et al. (2007) obtained a mean of 90.51% for the excretion of allantoin in dairy cows of average production (18.54 kg milk/day), being close to that observed in the present work, which also used cows with medium milk production. In dairy cows, allantoin and uric acid are also secreted in the milk, and the amount secreted daily is equivalent to, approximately, 5% of the PD excreted in urine (Chen & Gomes, 1992).

Table 7 – Urinary volume, urinary excretion of purine derivates, microbial protein synthesis and efficiency of microbial protein synthesis of dairy cows fed diets with or without (CON) the addition of propolis-based products (LLOS)

Parameters	Diets[1]				CV[2]	P
	CON	LLOS B1	LLOS C1	LLOS C3		
URV	16.85	16.57	18.12	19.38	14.83	ns
	Purine derivates					
ALA mmol/day	232.33	215.82	274.16	240.82	23.50	ns
UAc mmol/day	24.71	24.20	26.70	30.22	13.47	ns
MPD mmol/day	16.12	16.51	17.23	16.36	19.88	ns
PD mmol/day	273.16	256.52	318.09	287.39	20.06	ns
ALA%	84.67	83.78	85.97	83.15	4.08	ns
UAc%	9.25	9.59	8.47	10.73	16.16	ns
MPD%	6.08	6.63	5.56	6.12	33.10	ns
	Absorbed microbial purines (mmol/day)					
abMP	270.66	251.57	323.81	287.64	23.36	ns
	Microbial nitrogen compounds (g/day)					
micN	196.77	182.89	235.41	209.11	23.36	ns
	Microbial protein synthesis (g/day)					
MPS	1229.81	1143.06	1471.30	1306.99	23.36	ns
	EMPS (g MPS[3]/kg of TDN))					
EMPS[4]	111.52	104.56	126.76	116.71	22.96	ns

[1]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm [2]Coefficient of variation. [3]Grams of microbial protein. [4]Efficiency of microbial protein synthesis. URV: urinary volume (L/day); ALA: allantoin; UAc: uric acid; PD: purine derivates; MPD: milk purine derivates; ALA%, UAc% and MPD%:allantoin, uric acid and milk purine derivatives as % of total purine derivatives. ns: not significant.

The value found for the PD secreted in milk averaged 6.22%, slightly above that observed by the authors. The efficiency of microbial synthesis was not affected (P>0.05) by adding the products LLOS. According to the NRC (1996), a value of 130.0 g/kg of TDN for efficiency of microbial protein synthesis is a good estimate, but the treatment LLOS C1 was the only one closest to this estimate.

There was no influence (P>0.05) of the different LLOS on the evaluated blood parameters (Table 8). The same was observed by Faria et al. (2011), which found no effect of propolis (LLOS C1 in two increasing doses) on blood urea concentration in feedlot cattle, and also Simioni (2011), that reported no effect on blood parameters of feedlot cattle fed diets with higher doses than those used by Farias et al. (2011).

The concentration of MUN has become a useful tool in predicting the efficiency of N use in dairy cows (Burgos et al., 2007).

Table 8 - Means for concentrations of blood (BU), milk (MU) and urine urea (UU) and blood urea nitrogen (BUN), milk urea nitrogen (MUN), urine urea nitrogen (UUN) of dairy cows fed diets with or without (CON) the addition of propolis-based products (LLOS)

Parameters	Diets[1]				CV[2]	P
	CON	LLOS B1	LLOS C1	LLOS C3		
BU mg/dL	38.50	42.75	40.75	38.25	7.09	0.186
MU mg/dL	31.40	33.42	31.26	29.16	10.55	0.415
UU mg/dL	1790.0	1832.5	2000.0	1860.0	20.19	0.769
BUN mg/dL	17.99	19.98	19.04	17.87	7.09	0.186
MUN mg/dL	14.68	15.62	14.61	13.63	10.55	0.415
UUN mg/dL	836.45	856.31	934.58	869.16	20.19	0.769
MPr (%)[3]	3.76	3.77	3.63	3.61	18.05	0.980

[1]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm. [2]Coefficient of variation. [3]Milk protein.

The CP content of the diet is the most important nutritional factor that influences the MUN, and its determination can be used as a diagnosis in the use of dietary protein for dairy cows (Nousiainen et al., 2004) and the identification and correction of deficiencies, excess or imbalance of energy and protein diet (Godden et al., 2001). In this experiment, the mean for milk protein concentration was 3.69%, while that, for the MUN, it was obtained a mean concentration of 14.63 mg/dL. According to Godden et al. (2001), milk protein concentrations above 3.2% and MUN between 12-17 mg/dL, indicating proper balance of degradable protein and energy fermented in the rumen, and the values obtained for MUN and milk protein are within the desired.

For blood urea nitrogen (BUN), the treatments means was 18.72 mg/dL. Oliveira et al. (2001) found that BUN between 19-20 mg/dL and MUN between 24-25 mg/dL represent limits from which would be occurring N losses, and the values obtained for BUN and MUN are below the limits suggested by Oliveira et al. (2001).

Conclusions

The propolis-based products (LLOS) have positive effects on diet digestibility and ruminal parameters, because they promote more TD of DM, OM, CP and NDF. The LLOS B1 and C1 reduce CP ruminal digestibility, and the latter also reduces the NH_3 production; therefore, it is beneficial in dairy cows nutrition, since provides greater supply of amino acids into the small intestine and reduced N losses as NH_3.

However, depending on the concentration of propolis and alcoholic level used for propolis extraction, there will be changes in the active compounds extracted, as well as the amount of flavonoids and phenolic acids available, which can influence the microbial population from the rumen and, consequently, ruminal metabolism.

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CAPÍTULO V

(Normas: Revista Brasileira de Zootecnia)

Quality, Fatty Acid Composition and Antioxidant Capacity of Milk from Dairy Cows Fed Diets containing Propolis-Based Products

Abstract - The effects of propolis-based products (LLOS), with different concentrations of propolis (B and C) and alcohol levels (1 and 3), were evaluated on the quality, fatty acid (FA) composition and antioxidant capacity in milk samples from cows between 21 and 33 weeks of lactation. Four Holstein cows, weighing 550 ± 34.16 kg of BW and cannulated in the rumen were used, in a 4x4 Latin Square design (four treatments and four periods). The experimental diets (four) contained 59.19% of corn silage and 40.81% of concentrate, differing only in the absence (control diet) or presence of the LLOS (three products) with different concentrations of flavonoids. The propolis-based products did not affect (P> 0.05) the quality and somatic cell count of milk samples. It was observed an inversion in the milk fat and protein contents in all treatments, which was probably due to the addition of the soybean oil to diet. The addition of propolis affected (P <0.05) the FA composition and increased the antioxidant capacity of milk. At the lowest concentration of flavonoids there was an increase (P <0.05) in total polyunsaturated and monounsaturated FA, with reduction in the total saturated FA. At the highest concentration of flavonoids, there was a higher content of the *cis-9,trans-11* isomer (CLA) compared to the other treatments, followed by the intermediate concentration of flavonoids in the products. The addition of LLOS to the diet reduced the n-6/n-3 ratio when compared to control diet. For all LLOS, there was an increase (P <0.05) in the antioxidant capacity of milk in relation to control diet. It is concluded that the addition of propolis-based products in dairy cows' diet improved milk quality.

Keywords: additive, *Apis mellifera*, CLA, flavonoids, lipid oxidation

Introduction

There is increased consumer awareness that foods contain micro components that may have beneficial effects on health maintenance and disease prevention. In milk fat these functional food components include eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and conjugated linoleic acid (CLA). The opportunity to enhance the content of these FA in milk has improved as a result of recent advances that have better defined the interrelationships between rumen fermentation, lipid metabolism, and milk fat synthesis (Lock & Bauman, 2004).

Whole milk is over 96% fat-free, but on a dry basis, fat content is high (27%) with the majority (65%) of the fatty acids being saturated. About 50% of the calories in milk come from fat (Jenkins & McGuire, 2006).

The lipids of foods are subject to a series of reactions that can lead to modifications in their structures, affecting the nutritional value and also the quality standards, such as color, odor, flavor and texture (Donnelly & Robinson, 1995); and milk, which is rich in fat, is very susceptible to oxidation. The lipid oxidation is one of the major deteriorative reactions that occur during processing, distribution, storage and final preparation of food. It is responsible for the development of tastes and odors, making the food unfit for consumption. It also causes other changes that will affect the nutritional quality, integrity and safety of food (Soares, 2002); therefore, much research is focused on the search for alternatives to reduce the process of lipid oxidation in food.

Propolis is a resin collected by bees from plants, being mixed with wax and used in the construction and adaptation of their hives (Bankova et al., 2000). Because of the diversity in their chemical composition and its biologically active components (flavonoids and phenolic acids, mainly), propolis exerts numerous pharmacological activities, such as antimicrobial and antioxidant (Loghercio et al., 2006, Laskar et al., 2010), and can be an alternative for production of functional foods as a natural additive in ruminant nutrition, for example. Many studies have found that the potential antioxidant activity of propolis is directly related to the concentration of phenolic compounds in the extracts, which are influenced by the extraction conditions (concentration of propolis and alcohol content) (Kumazawa et al., 2004, da Silva et al., 2006; Cottica et al., 2011).

Therefore, the objective was to evaluate the fatty acid composition and antioxidant capacity in milk samples from cows fed diets containing propolis-based products (LLOS).

Material and Methods

The experiment was carried out in the city of Maringá, Paraná state, Brazil. Four primiparous Holstein cows were used, with 147 days of lactation, weighing 550 ± 34.16 kg of body weight (BW), cannulated in the rumen, housed in individual cages and subjected to two daily milkings (6h and 15h). The animals were randomly assigned to a 4 x 4 Latin square, with four periods and four treatments. The propolis-based products differed in the concentration of propolis (B and C, between 5.0 and 30.0% (w/v)) and water-alcohol solutions (1 and 3, between 60.0 and 93.8% (v/v)), prepared according to the methodology developed by Franco & Bueno (1999). The propolis samples were obtained from the apiary of the Experimental Farm of Iguatemi (FEI), belonging to the Universidade Estadual de Maringá, Paraná State, Brazil, being certified as organic. The apiary is located within a reserve of eucalyptus (*Eucalyptus* sp.) surrounded by native forest, with the presence of alecrim-do-campo (*Baccharis dracunculifolia*).

Propolis-based product (LLOS), a powder, contains dried propolis extract and is registered in the National Institute of Industrial Property – Brazil, under no. 0605768-3. The preparation of LLOS consists of the hydroalcoholic extraction of raw propolis to release its active substances – flavonoids, mainly. Subsequently, the alcohol is evaporated with the aid of a rotary evaporator and the extract is dried. Due the amount of extract supplied to the animals is too small, was added to the extract an excipient (corn and soybean meal, 50:50) to add volume facilitate the animal feeding. The daily amount of some flavonoids and phenolic acids provided to the animals (through the LLOS products) is shown in Table 1. The quantification of these compounds was performed using high-performance liquid chromatography (HPLC).

The experimental diets containing, 59.19% of corn silage and 40.81% of concentrate, differed with the inclusion or not of the LLOS products constituting, therefore, in four treatments: control (no additive), LLOS B1, LLOS C1 and LLOS C3.

Table 1 – Composition in flavonoids and phenolic acids identified in the propolis-based products (LLOS)[1] supplied daily for dairy cows

	Propolis dry extract[1]		
	LLOS B1	LLOS C1	LLOS C3
	mg/g of propolis dry extract		
Chlorogenic acid	n.f.	0.24	n.f.
Caffeic acid	4.06	4.75	3.10
p-coumaric acid	7.16	8.15	5.27
Benzoic acid	0.59	1.20	0.45
CAPE[2]	2.73	2.68	1.49
Artepillin C	7.59	7.27	4.62
Apigenin	7.66	5.69	3.72
Pinocembrin	4.92	3.62	2.33
Galangin	1.49	n.f.	n.f.
Chrysin	3.90	2.65	1.61
Acacetin	4.06	3.65	2.04
	g of LLOS/kg of ingested dry matter		
Total flavonoids	2.81	2.14	1.22
Artepillin C and CAPE	0.40	0.41	0.25
Total phenolic acids[3]	1.00	1.13	0.71

n.f. = not found. [1]Concentrations of propolis (B and C) between 5.0 and 30.0% (w/v) in water-alcohol solutions (levels 1 and 3) between 60.0 and 93.8% (v/v) of alcohol. [2]Caffeic acid phenethyl ester. [3]Sum of the phenolic acids grouped at the beginning of the chromatogram with CAPE and Artepillin C.

The chemical composition of the experimental diet is presented in Table 2, which was formulated according the recommendations proposed by NRC (2001) for lactating cows with approximately 550 kg BW, 21 weeks of lactation and with estimated milk production of 25.0 kg, with 3.8% fat. The net energy for lactation (NEL) was estimated using the equation: NEL (Mcal/kg) = 0.0245 x %TDN – 0.12 (NRC, 2001), obtaining the value of 1.63 Mcal/kg.

The animals were fed twice daily, at 8h and 16h, with forage and concentrate mixed on the trough. All animals received the same experimental diet, differing only in the addition of propolis or not (control).

The propolis-based products were placed into the rumen via ruminal cannula at the time of feeding. The animals received two daily doses of LLOS (7.5 g) previously weighed in hygroscopic paper, with a total of 15.0 g of LLOS/day.

The analysis to determine dry matter (DM, method no. 934.01), organic matter determined by ash (OM, method no. 924.05), crude protein (CP, method no. 920.87) and ether extract (EE, method no. 920.85) in the samples milled to 1 mm, were conducted in accordance to the AOAC (1990).

Table 2 – Chemical composition and proportion of ingredients used in the experimental diet

	g/kg[1]									
	DM	OM	CP	EE	NDF	ADF	TC	NFC	TDN	Diet (%)
Corn silage	292.7	962.3	72.7	30.3	606.7	337.1	856.2	249.6	634.4	59.19
Soybean meal	898.0	935.1	462.1	14.9	182.3	100.4	433.2	250.9	806.8	19.77
Ground corn	878.7	985.1	91.2	18.3	165.2	37.6	869.0	704.0	832.0	5.26
Wheat meal	857.1	948.1	170.7	23.2	458.7	148.8	754.2	295.5	715.4	10.48
Soybean oil	995.7	997.0	-	991.3	-	-	-	-	2139	2.86
V.M. suppl.[2]	990.0	-	-	-	-	-	-	-	-	1.98
Limestone	991.4	-	-	-	-	-	-	-	-	0.32
Am.sulfate	990.0	-	1250	-	-	-	-	-	-	0.14
Exp. diet	539.4	934.1	160.6	52.60	451.8	236.9	722.4	270.6	714.9	100.0

[1]DM= dry matter, OM= organic matter, CP= crude protein, EE= ether extract, NDF= neutral detergent fiber, ADF= acid detergent fiber, TC= total carbohydrates, NFC= non-fiber carbohydrates, TDN= total digestible nutrients. [2]Composition of vitamin and mineral supplement (per kg of product): 146 g of calcium, 51 g of phosphorus, 20 g of sulfur, 33 g of magnesium, 93 g of sodium, 28 g of potassium, 30 mg of cobalt, 400 mg of copper, 10 mg of chromium, 2.000 mg of iron, 40 mg of iodine, 1.350 mg of manganese, 15 mg of selenium, 510 mg of fluoride, 1.700 mg of zinc, 135.000 IU of Vit. A, 78.000 IU of Vit D3 and 450 IU of Vit. E.

Neutral detergent fiber (NDF) was determined according to Van Soest et al. (1991) and acid detergent fiber (ADF) determined according to method no. 973.18 (AOAC, 1990). The total carbohydrates (TC) were obtained by using the following equation: TC = 100 - (% CP +% EE +% Ash), (Sniffen et al., 1992). Non-fiber carbohydrates (NFC) were determined by the difference between TC and NDF (without correction for protein). TDN content of the experimental diets was calculated using the following equation: %TDN = %DCP + %DNDF + %DNFC + %(DEE x 2.25), where: DCP = digestible crude protein, DNDF: digestible neutral detergent fiber, DNFC = digestible non-fiber carbohydrates, DEE = ether extract.

The experiment consisted of four experimental periods of 21 days each. For the analysis of physico-chemical composition of milk, samples were collected in the 17th and 18th days of each experimental period, during the morning and afternoon milkings (6 h and 15 h). Milk yield was recorded daily, to monitor the performance of animals. Milk yield was also corrected to 3.5% of fat (FCM) for further evaluation, according to the equation described by Sklan et al. (1994): FCM = milk yield (kg/day) × (0.432 + 0.163 fat %).

The acidity of milk was obtained by Dornic technique and density was determined by the thermolactodensimeter of Quevenne (AOAC, 1990). For chemical

analysis of milk, samples were analyzed for determination of fat, protein, lactose and total solids by infrared absorption through the analyzer 2000 Bentley®, and somatic cell count (SCC) were analyzed through cytometric flowmeter, in an electronic counter Somacount 500®.

The total lipids of the milk samples were extracted in triplicate according to Folch et al. (1957) and, for esterification and transesterification, they were subjected to methylation process, as described by Hartman & Lago (1973) and modified by Maia & Rodrigues-Amaya (1993).

The esters of fatty acids were analyzed by a Thermo Scientific gas chromatograph (Thermo Fisher Scientific Inc., USA), model Trace GC Ultra equipped with an autosampler TriPlus, with a flame ionization detector and a fused silica capillary column CP-7420 (100 m, 0.25 mm i.d. and 0.39 mM, 100% cyanopropyl) (Martin et al., 2008). The gases flow (White Martins, Praxair Technology Inc., USA) were 1.4 mL/min for the carrier gas (H_2); 30 mL/min for the auxiliary gas (N_2); 30 mL/min and 300 mL/min for the H_2 and the synthetic air flame, respectively. The division ratio of the sample (split) was 1/80. The temperatures of the injector and detector were 230°C and 240°C, respectively. The column temperature was programmed at 65°C for 4 minutes, followed by a first ramp of 16°C/min until 185°C, remained for 12 minutes. A second ramp was programmed from 20°C/min to 235°C, kept at this temperature for 9 minutes. The total time of analysis was 35 minutes. The peak areas were determined by ChromQuest software, version 5.0. The injections were performed in duplicate and the injection volumes were 2 μL. The FA identification was based on comparison of retention times with those of standards of fatty acid methyl esters, and the results were expressed as percentage of relative area, by the normalization method (Visentainer et al., 2012).

The analysis of antioxidant capacity of milk samples was performed using the ORAC (Oxygen Radical Absorbance Capacity) method, which evaluates the antioxidant capacity of the sample, measuring its ability to protect the fluorescein (FL) from the oxidation by 2,2′- azobis-(2-amidinopropane) dihydrochloride (AAPH) in the reaction, as described by Zulueta et al. (2009) with some modifications. The entire procedure was conducted at 37° C and used potassium phosphate buffer (pH 7.0) as solvent. Milk samples were previously thawed and homogenized in ultrasonic bath, and diluted in a

volumetric flask with 50 mL phosphate buffer to a dilution of 1:500 (v/v). Then, a further dilution was performed, resulting in a final dilution of 1:1250 (v/v).

The blank was prepared by mixing 250 µL of phosphate buffer with 1500 mL of the FL solution at 4.0 nmol/L, prepared from a stock solution (1.0 mmol/L), and 250 mL of AAPH solution at 160 mmol/L. By reading the fluorescence intensity (excitation = 485 nm, emission = 515 nm), it was calculated the area under the curve of absorbance *versus* time. The readings were taken at intervals of 1 minute, during 30 minutes, in spectrofluorimeter. For samples evaluation, it was added 250 µL of extract solution previously prepared in quartz cuvette with 1500 µL of FL solution at 4.0 nmol/L and 250 mL of AAPH solution at 160 mmol/L, repeating the measurements made with the blank and, to the calibration curve, was added 250 µL of Trolox solution (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) to the same quantities of the FL and AAPH solutions previously used. The incubation temperature of the reaction was 37°C, so that the reaction with free radicals consumes FL and the fluorescence decay. The results were expressed in µmol of Trolox equivalent (TE/L), calculated by the linear equation obtained from the calibration curve with the Trolox standard ($r^2 = 0.9884$):

$$y = 0.7837 + 0.1545x,$$

where x is the ORAC value expressed in µmol TE/L and y is the area under the curve fluorescence decay (AUC) of sample or standard subtracting the AUC of the blank, and the AUC can be obtained according to the following equation:

$$AUC = (1 + f_1/f_0 + f_2/f_0 + \ldots + f_{n+1}/f_0),$$

where f_0 is the initial fluorescence intensity and f_n is the fluorescence intensity at time n.

The data were interpreted by analysis of variance using the MIXED procedure of SAS statistical software (2001). Due to not follow a normal distribution, the value of SCC were converted into the logarithm (log) in base 10. The mathematical model used for the analysis was: $Y_{ijk} = \mu + A_i + P_j + T_k + e_{ijk}$, where: Y_{ijk} = observed variables, μ = overall mean, A_i = effect of animal i, ranging from 1 to 4; P_j = effect of the period j

varying from 1 to 4; T_k = k effect of the treatment, ranging from 1 to 4; e_{ijk} = random error.

Results and Discussion

The addition of products LLOS in diet did not affect (P>0.05) the yield and milk composition of cows (Table 3).

Table 3 – Yield, fat corrected milk (FCM) and milk composition of cows fed diets with or without (CON) the addition of propolis-based products (LLOS)

	Diets[1]				CV[2]	P
	CON	LLOS B1	LLOS C1	LLOS C3		
Yield (kg/day)	16.12	17.23	16.36	16.51	3.63	0.152
FCM (kg/day)	14.41	14.40	14.51	14.74	5.59	0.536
Fat (%)	2.87	2.50	2.79	2.85	8.59	0.195
Protein (%)	3.76	3.77	3.63	3.61	6.21	0.487
Lactose (%)	4.40	4.41	4.35	4.45	2.03	0.658
Total solids (%)	12.00	11.69	11.70	11.92	2.86	0.547
Acidity ^oD	17.21	17.66	16.43	17.72	6.04	0.353
Density	1.03	1.03	1.03	1.03	0.05	0.136
SCC ($x10^3$/mL)	204.63	145.94	193.25	164.81	29.96	0.450
SCC[1]	2.15	2.00	2.07	2.07	3.92	0.174

[1]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm. [2]Coefficient of variation.[3]CCS = somatic cell counts log_{10}.

There are few studies involving the effects of propolis on production and milk composition. Lana et al. (2005) tested soybean oil and/or ethanolic propolis extract in diets of dairy goats on milk yield and composition and found no changes (P>0.05) on the parameters evaluated with the addition of the propolis extract. Subsequently, Stelzer et al. (2009) evaluated two levels of concentrate (20 and 40% of DM) and the presence or absence of ethanolic propolis extract (30% w/v) in dairy cows diet and also found no effect of the propolis extracts on the production and milk composition. However, Freitas et al. (2009) observed that the addition of ethanolic propolis extract increased (P<0.05) production and protein content of milk from Holstein cows.

However, there was a reversal in milk fat and milk protein, regardless of diet. Diets with high content in grains and low forage can cause milk fat depression (MFD) due to the low fiber content. Another factor that may cause MFD is the addition of polyunsaturated oils in the diet (vegetable oils, for example). In fact, the presence of polyunsaturated FA (PUFA) is a prerequisite for MFD occurs in animals fed diets with

low fiber content (Griinari & Bauman, 2003). Under certain dietary conditions the pathways of rumen biohydrogenation are altered to produce unique fatty acid intermediates which are potent inhibitors of milk fat synthesis (such as *trans*-10,*cis*-12 and *trans*-10-18:1 isomers). In diets containing vegetable oils, the isomer *trans*-10,*cis*-12 became a major intermediate. High concentrations of *trans*-10-18:1 occur in digesta and consequently in the FA flowing to animal tissues and, under these circumstances, milk fat depression occurs (Bauman & Griinari, 2001; Lourenço et al., 2010). In other studies using soybean oil in dairy cows diets also decreased milk fat (Mohamed et al., 1988; Eifert et al., 2005; Eifert et al., 2006a; AlZahal et al., 2008; Santos et al., 2009), confirming the results obtained in this study.

There was no influence ($P>0.05$) of the propolis-based products on SCC (Table 3), although *in vitro* studies that tested different propolis extracts on the sensitivity of the causative agents of mastitis (*Staphylococcus* sp. and *Streptococcus* sp.) found in cattle and goats, observed inhibition in growth of these bacteria (Pinto et al., 2001; Loghercio et al., 2006, Santos Neto et al., 2009). According to data obtained in this work, perhaps at higher doses, the propolis-based products could allow possible effect of reducing the number of somatic cells in milk, given that these were numerically lower in the milk of cows that received propolis.

The FA composition of the milk samples was influenced ($P<0.05$) by the addition propolis-based products (Table 4). It can be observed that the propolis decreased ($P<0.05$) the content of short chain fatty acids (4:0, 6:0 and 8:0) compared to the control diet, whereas for 4:0 and 6:0, there was a greater reduction for the diet containing LLOS B1 and, for 8:0, the LLOS C3 showed the lowest content. Values greater than those obtained in this study for short chain FA (2.00% for 4:0, 1.62% for 6:0 and 1.23% for 8:0), using the same method of lipids extraction was observed by Tonial et al. (2009), who evaluated different lipid extraction methods on fatty acid composition of milk from cows (whole milk).

Reductions in of short chain fatty acid content in milk from cows supplemented with soybean oil are reported in the literature (Mohamed et al., 1988, Santos et al., 2001; Eifert et al., 2006b; AlZahal et al. 2008).

Table 4 – Fatty acids composition (percentage of relative area) of milk samples from cows fed diets with or without (CON) the addition of propolis-based products (LLOS)

Fatty acid	Diets[1]				CV[2]	P
	CON	LLOS B1	LLOS C1	LLOS C3		
4:0	1.036a	0.795c	0.865bc	0.972ab	7.090	0.004
6:0	1.100a	0.898b	0.961ab	1.057a	7.300	0.013
8:0	0.745a	0.693ab	0.668b	0.483c	4.940	<0.001
10:0	1.878a	1.743b	1.693b	1.201c	3.800	<0.001
11:0	0.029ab	0.027b	0.028ab	0.030a	5.340	0.029
12:0	2.357	2.257	2.189	2.153	4.560	0.132
13:0	0.094	0.102	0.088	0.091	8.140	0.279
13:1n-5	0.142b	0.120c	0.118c	0.172a	2.150	<0.001
14:0	8.222	8.309	8.031	7.511	5.560	0.433
14:1n-5	1.208a	1.162a	0.928b	0.963b	4.490	<0.001
15:0	0.925	0.920	0.914	0.832	9.000	<0.001
15:1n-5	0.295b	0.274b	0.289b	0.356a	8.250	0.006
16:0	20.845	21.619	21.614	19.511	4.530	0.286
16:1n-7	1.355c	1.838ab	1.937a	1.704b	3.830	<0.001
16:1n-5	0.567a	0.450b	0.519ab	0.452b	6.470	0.005
17:0	0.477	0.456	0.459	0.389	8.380	0.112
17:1n-7	0.229b	0.252ab	0.269a	0.261a	7.010	0.060
18:0	10.387ab	10.059bc	10.728a	9.121c	3.200	0.005
18:1n-9*t*	5.164b	6.170a	4.977b	4.994b	5.340	0.006
18:1n-9	36.328b	36.170b	36.226b	40.774a	4.040	0.002
18:2n-6 (LA)[3]	4.914a	3.865c	4.277b	4.947a	3.170	<0.001
18:3n-3 (ALA)[4]	0.475b	0.482b	0.511b	0.631a	4.320	<0.001
18:2n(9*c*,11*t*) (CLA)[5]	0.789c	0.886c	1.205a	0.953b	4.670	<0.001
20:0	0.184ab	0.169bc	0.203a	0.147c	6.860	0.004
18:2n(10*t*,12) (CLA)	0.047c	0.072b	0.072b	0.085a	3.550	<0.001
20:3n-6	0.079b	0.083ab	0.092a	0.078b	5.490	0.027
20:4n-6 (AA)[6]	0.118	0.115	0.126	0.120	8.720	<0.001

Different letters in the same line are statistically different (P<0.05) by Tukey test. [1]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm. [2]Coefficient of variation.[3]LA = linoleic acid, [4]ALA = alpha-linolenic acid, [5]CLA = conjugated linoleic acid, [6]AA = arachidonic acid.

The fatty acids in milk arise from two sources, uptake from circulation and *de novo* synthesis within the mammary epithelial cells (Bauman & Griinari, 2003). Substrates for *de novo* synthesis are acetate and β-hydroxybutyrate derived from rumen fiber digestion. They are used by the mammary epithelial cell to synthesize short- and medium-chain FA (4:0 to 14:0) plus a portion of the 16-carbon FA (Lock & Bauman, 2004).

However, FA uptake from circulation are derived from diets containing sources of fat rich in PUFA, which reduces *de novo* synthesis to a greater extent so that the milk fat content of short and medium chain fatty acids is reduced and the content of longer chain fatty acids is incresed (Bauman & Griinari, 2001).

Regarding the medium chain (10-14 carbons) and odd chain FA, the LLOS altered ($P<0.05$) the content of 10:0 and 11:0, but there was no significant difference between treatments ($P>0.05$) for 12:0, 13:0, 14:0, 15:0 and 17:0. The LLOS C3 provided the lowest content of 10:0, and together with LLOS C1 reduced the content of 14:1n-5. The values found are much lower than those observed by Tonial et al. (2009), where 10:0, 12:0 and 14:0 showed means of 2.44, 2.78 and 12.36%, respectively. This reduction confirms the values obtained by Santos et al. (2001), Eifert et al. (2006b) and AlZahal et al. (2008), who used soybean oil in diet, and that was probably caused by a reduction in *de novo* synthesis. The lowest content of 12:0 and 14:0 FA in milk is desirable, since these possess hypocholesterolemic effects (Bauman & Griinari, 2001).

The long chain FA (>16 carbons) 18:0 and 20:0 present in milk fat were influenced by the addition of the propolis-based products ($P<0.05$) in the diet (Table 4) and LLOS C3 reduced the content of 18:0 and 20:0 FA. Although the 18:0 is saturated fatty acid (obtained through the biohydrogenation of unsaturated FA) its reduction is undesirable, because it acts to reduce blood cholesterol, which is important for human health (Kenelly, 1996).

Regarding linoleic (LA) and alpha-linolenic (ALA) fatty acids it appears that, for linoleic content, there was a reduction ($P<0.05$) for LLOS B1 and C1, and LLOS C3 did not differ from control. As for ALA content, an increase ($P<0.05$) was observed for the lower concentration of flavonoids (LLOS C3). The LA content obtained is much higher than the observed in commercial milk (1.53%), according to Tonial et al. (2009). This is due to the addition of the fat source to the experimental diet, because the soybean oil is rich in LA that, in the form of triglycerides, is the major substrate for

ruminal biohydrogenation. However, the extent of lipolysis and rumen biohydrogenation decrease with increasing amounts of substrate, that is, much of the LA escapes biohydrogenation, increasing the content of fatty acids in milk. Also, in these conditions, bacteria and protozoa may incorporate LA and other FA in its membrane structure, inhibiting the *de novo* synthesis and, thereby, increasing the content of long chain fatty acid in milk (Eifert et al., 2006b, Lourenço et al., 2010).

According to Eifert et al. (2006b), changes in rumen biohydrogenation pathways, from a microbiological point of view, may be a consequence of the stimulation or inhibition in the growth of certain species or groups of rumen bacteria, either by dietary factors (carbohydrate degradation rate, ruminal pH, preferred substrate) or, in this case, with the addition of propolis, antimicrobial action. The LLOS can act on certain rumen bacteria involved in rumen biohydrogenation, changing thereby, the composition of milk FA, as shown in Table 4.

The content of conjugated linoleic acid (CLA) was influenced ($P<0.05$) by the LLOS. The LLOS B1 had the highest content of 18:2n (*cis*-9,*trans*-11) compared to other treatments, followed by LLOS C3. Regarding the 18:2n (*trans*-10,*cis*-12), the LLOS C3 had the highest content followed by other products LLOS, and the control showed the lowest content. The values obtained for the *trans*-10,*cis*-12 isomer are much higher than the normally found in milk. Some authors found minimum or even did not detect this isomer in milk in diets with or without the addition of oil (Collomb et al., 2004; Eifert et al., 2006a; Eifert et al., 2006b; Bell et al. , 2006; Loor & Herbein, 2003). The high content of *trans*-10,*cis*-12 may be one of the causes of milk fat depression, as previously discussed. The anti-obesity effects of CLA are due to the *trans*-10,*cis*-12 isomer; while this isomer can vary in milk fat, it never represents more than 1 or 2% of total CLA, and food products derived from ruminants are thus unlikely to provide sufficient amounts of this isomer to have biological effects on body fat (Lock & Bauman, 2004); however, it appears that the LLOS increased CLA in milk, which may be considered a positive effect, even though its concentration in milk is very small.

The intake of CLA in humans is of great interest due to the great health benefits that they may confer, as its anticancer property, for example. The predominant source of CLA in human diets is foods derived from ruminants, with dairy products contributing approximately with 75% of the total. CLA is a component of milk fat, therefore, research has focused on increasing the content of CLA *per* unit of fat (Lock & Bauman,

2004). The CLA isomer *cis*-9,*trans*-11 is only formed during the biohydrogenation of linoleic acid and is the first intermediate in biohydrogenation pathway, while vaccenic acid is an intermediate formed both from linoleic and linolenic acid. Vaccenic acid and CLA are both present in ruminant fat, and it was generally assumed they were of rumen origin and represented intermediates that had escaped complete biohydrogenation. However, *cis*-9,*trans*-11 CLA is only a transitory intermediate in rumen biohydrogenation, whereas vaccenic acid tends to accumulate; and the main source of CLA in milk fat is from endogenous synthesis. An important discovery within the last years was the observation that the Δ^9-desaturase (an enzyme that introduces a *cis*-9 double bond in fatty acids) was the predominant source of the *cis*-9,*trans*-11 CLA isomer in milk, which has a number of benefits to human health (including anticarcinogenic properties). Vaccenic acid arising from biohydrogenation in the rumen is transferred to the mammary tissue and desaturated to *cis*-9,*trans*-11 CLA via the Δ^9-desaturase. This has shifted attention to manipulating ruminal biohydrogenation to enhance the yield of the *trans*-11 isomer (Bauman & Griinari, 2001; Lock & Bauman, 2004; Jenkins & McGuire, 2006). The propolis-based products (LLOS) C1 and C3 increased by 52.7% and 20.8% the content of *cis*-9,*trans*-11 compared to control, respectively. These data show that propolis modifies rumen biohydrogenation, and may be inhibiting the reduction of vaccenic acid to stearic acid, which increases the escape of vaccenic acid from rumen, allowing more endogenous synthesis in the mammary gland. The LLOS C3 increased (P<0.05) total PUFA and MUFA and reduced total SFA, as shown in Table 5.

Table 5 – Sum of fatty acids (percentage of relative area) of milk samples from cows fed diets with or without (CON) the addition of propolis-based products (LLOS)

	Diets[1]				CV[2]	P
	CON	LLOS B1	LLOS C1	LLOS C3		
PUFA[3]	6.424b	5.505c	6.285b	6.816a	2.380	<0.001
SFA[4]	48.283a	48.052ab	48.446a	43.504b	2.820	0.029
MUFA[5]	45.291b	46.441b	45.267b	49.678a	3.630	0.006
n-6	5.160a	4.136c	4.569b	5.231a	3.040	<0.001
n-3	0.475b	0.482b	0.511b	0.631a	4.320	<0.001
n-6/n-3	10.855a	8.579b	8.933b	8.280b	4.910	<0.001
Total CLA[6]	0.837c	0.958c	1.277a	1.038b	4.280	<0.001

Different letters in the same line are statistically different (P<0.05) by Tukey test. [1]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm. [2]Coefficient of variation. [3]PUFA = polyunsaturated fatty acids, [4]SFA = saturated fatty acids, [5]MUFA = monounsaturated fatty acids, [6]Total CLA = sum of conjugated linoleic acid isomers (CLA).

Regarding the n-6/n-3 ratio, the products LLOS reduced (P<0.05) significantly this reason, which is desirable. The content of n-3 fatty acids (EPA, 20:5n-3 and DHA, 22:6n-3) in milk fat is of great interest, due to the benefits they provide to human health. These n-3 FA reduce the risk of cardiovascular disease, diabetes type 2, hypertension, cancer and certain destructive neurological functions (Simopoulos, 1991; Connor, 2000).

Milk samples from cows that received the propolis-based products in diet showed higher (P=0.000001) antioxidant capacity compared to control, as shown in Table 6.

Table 6 – Antioxidant capacity of milk samples from cows fed diets with or without (CON) the addition of propolis-based products (LLOS)

Diets[1]	Antioxidant capacity (TE µmol/L)
CON	14581.73±928.17c
LLOS B1	23640.26±1116.54a
LLOS C1	24352.31±1451.56a
LLOS C3	16074.84±1435.41b
CV[2]	2.88

Different letters in the same line are statistically different (P<0.05) by Tukey test. [1]LLOS B1 = 3.81 ppm, LLOS C1 = 3.27 ppm, LLOS C3 = 1.93 ppm. [2]Coefficient of variation.

The products LLOS B1 and C1 with higher concentrations of flavonoids (2.81 and 2.14 ppm, respectively) and phenolic acids (1.00 and 1.13 ppm, respectively) were more efficient in increasing the antioxidant capacity of milk samples, followed by LLOS C3 (1.22 and 0.71 ppm of flavonoids and phenolic acids, respectively).

Many studies investigated the antioxidant capacity of flavonoids and phenolic acids singly. Among flavonoids, we highlight the galangin (Russo et al., 2002, Laskar et al., 2010) and, for phenolic acids, caffeic acid, *p*-coumaric acid, caffeic acid phenethyl ester (CAPE) and Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), and the latter two have high antioxidant capacity (Vieira et al., 1998, Russo et al, 2002; Hoşnuter et al., 2004, Shimizu et al., 2004). Geckil et al. (2005) found that propolis has features such as free radical scavenging and metal chelation. The mechanisms of antioxidant action may include: (1) suppress the formation of reactive oxygen species, either by enzymatic inhibition or chelating elements involved in the production of free radicals;

(2) elimination of reactive oxygen species (ROS) and (3) regulating or protecting antioxidant defenses. Studies have shown that flavonoids satisfy most of the items described above (Pietta, 2000).

The ROS are formed continuously in cells as a consequence of oxidative biochemical reactions and external factors. However, they become harmful when produced in excess under certain abnormal conditions (ischemia, inflammation and in the presence of catalytic iron ions). Under these conditions, the endogenous antioxidants may be unable to prevent the formation of ROS that, when excessive, can cause cell damage involved in several diseases (coronary heart disease, inflammation and neurodegenerative diseases (like Parkinson's and Alzheimer's disease) and cancer) (Pietta, 2000; Russo et al., 2002).

Russo et al. (2002) evaluated the antioxidant capacity of propolis extracts with (10.44%) or without CAPE and found that propolis extracts containing CAPE showed higher capacity to eliminate free radicals and greater ability to inhibit the activity of xanthine oxidase (which is a physiological source of superoxide anions in eukaryotic cells) than the extract without CAPE. In this study, it appears that products with antioxidant capacity (LLOS B1 and C1) contain higher levels of CAPE and Artepillin C than LLOS C3, and this showed the lowest antioxidant capacity among all propolis-based products.

In *in vitro* studies, it was found that flavonoids may bind to milk protein, but this was not confirmed in *in vivo* studies (Pimentel et al., 2005). According to Galleano et al. (2010), alterations in membrane and protein functions can happen at very low flavonoid concentrations, and have major effects on biological events. Then, lipid–flavonoid or protein–flavonoid interactions can be relevant at concentration much lower than those necessary to cope with a constant free radical production. In terms of inhibition of free radical formation, the superoxide dismutase activity, the inhibition of NADPH oxidase activity, and the modulation of nitric oxide synthase are examples of ‘‘indirect” antioxidant effects derived from flavonoid–protein interactions.

Conclusions

The propolis-based products (LLOS) have effect on milk fat composition. The LLOS C3, with lower concentrations of phenolic compounds, increased total PUFA and

MUFA, and reduced SFA content. The LLOS increased *cis*-9,*trans*-11 content and reduced the n-6/n-3 ratio. All LLOS inhibited the progress of lipid oxidation in milk, and this was greater for LLOS with higher concentrations of phenolic compounds.

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CAPÍTULO VI

(Normas: Current Microbiology)

Antimicrobial activity of different Brazilian propolis extracts against rumen bacterial strains

Abstract – The antimicrobial activity of Brazilian propolis extracts, obtained from different extracts conditions, was evaluated on rumen bacterial strains. The extracts differed in the concentration of propolis and alcohol level, resulting in extracts with different phenolic compositions (B1 = 148.13, C1 = 121.13 and C3 = 70.27 mg of phenolic compounds g^{-1} of propolis extract). Some of the phenolic compounds present in the propolis extracts (naringenin, chrysin, caffeic acid, *p*-coumaric acid and Artepillin C) were also evaluated on the strains. The assay was conducted in Hungate tubes (4 ml of the growth medium) with different concentrations of propolis (250, 500 and 1000 µg ml^{-1}) and the control tubes contained the growth medium and ethanol at 80%. After the incubation period (15 h), bacterial growth was monitored using turbidimetry. The different propolis extracts inhibited the growth of *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *R. albus* 7, *Butyrivibrio fibrisolvens*, *Prevotella albensis*, *P. ruminicola*, *Peptostreptococcus* sp., *Clostridium aminophilum* and *Streptococcus bovis*, while *R. albus* 20, *P. bryantii* and *Ruminobacter amylophilus* were resistant to all the extracts. The potential of inhibition was influenced by the extraction conditions of the phenolic compounds present in propolis. Among the isolated phenolic compounds, only naringenin had inhibitory effect against all strains, suggesting that the antimicrobial activity of propolis is due to a synergism between its components. The study of the biological properties of propolis should be linked to a detailed investigation of its chemical composition, to better understand its effects.

Keywords: antibacterial activity, phenolic compounds, propolis extraction, rumen bacteria

Introduction

Propolis, or bee glue, is a resinous material collected by worker bees from the leaf buds of numerous tree species. Once collected, this material is enriched with salivary and enzymatic secretions and is used by bees to cover hive walls, fill cracks or gaps and embalm killed invader insects [3]. Propolis presents plenty of biological and pharmacological properties, such as immunomodulatory, antitumor, antiinflammatory, antioxidant, antibacterial, antiviral, antifungal, antiparasite activities, among others [19, 9, 7, 1]. This biological potential is due to a synergism among its many constituents [11]; especially the phenolic compounds (flavonoids and phenolic acids). However, propolis chemical composition depends on the specificity of the local flora at the site of collection and thus on the geographic and climatic characteristics of this site. This fact results in the striking diversity of propolis chemical composition [2]. Furthermore, the propolis cannot be used in its raw form and may be purified by extraction with solvents, in order to remove inert material and preserve the polyphenolic fraction. In addition, depending on the type and quantity of solvent employed, as well as the concentration of propolis used for the extraction, the propolis extracts may have different chemical compositions and different biological activities [23, 4, 19].

Due to its antimicrobial activity, propolis has also been studied in ruminant nutrition, as an alternative to antibiotics or chemical additives used to increase the production of these animals. These studies showed that propolis was effective in increasing dry matter and nutrient digestibility in buffalos and the protein flow into the small intestine in cattle, besides inhibiting ammonia production *in vitro* [14-16]. Although there are numerous studies that investigated the antimicrobial activity of propolis, little is known about its antimicrobial activity against rumen microbiota.

Therefore, the objective was to evaluate the antimicrobial activity of Brazilian propolis extracts and some of its isolated compounds on rumen bacteria and verify if the different extraction conditions may influence their potential for microbial inhibition.

Material and Methods

Propolis samples

The propolis samples were obtained from the apiary of the Experimental Farm of Iguatemi (FEI), belonging to the Universidade Estadual de Maringá, Paraná State, Brazil. The apiary is located within a reserve of eucalyptus (*Eucalyptus* sp.) surrounded by native forest, with the presence of alecrim-do-campo (*Baccharis dracunculifolia*). The propolis samples were obtained from colonies of africanized honeybees (*Apis mellifera*) and were placed in plastic containers and stored at a freezing temperature of -22^{o}C.

Preparation of the propolis extracts

The propolis dry extracts (LLOS) were prepared according to the methodology described by Franco and Bueno [6] and were obtained using two increasing concentrations of propolis (5 to 30% w/v) named B and C, and two increasing alcohol levels (60 to 96% v/v) named 1 and 3, resulting, therefore, in three different propolis dry extracts, named B1, C1 and C3. The information on the concentrations of propolis and alcohol levels is protected by intellectual property application under nº. 0605768-3, in Brazil. The amount of phenolic compounds present in the extracts was obtained using high performance liquid chromatography (HPLC), where 148.13, 121.13 and 70.27 mg of phenolic compounds g^{-1} of propolis extract were quantified for LLOS B1, C1 and C3, respectively. For the evaluation of antimicrobial activity, the dry propolis extracts were diluted in 80% of ethanol at a concentration of 100 µg of propolis ml^{-1} of solution, then filtered and stored protected from light.

Bacterial strains

Twelve bacterial strains, belonging to the major bacterial species and functional groups from the rumen, were tested for antimicrobial activity of the different propolis extracts. These were five fibrolytic strains (*Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, *Ruminococcus albus* 7, *Ruminococcus albus* 20 and *Butyrivibrio fibrisolvens* DSMZ 3071), four proteolytic strains (*Prevotella albensis* DSMZ 11370, *Prevotella ruminicola* 11370, *Peptostreptococcus* sp. D1 and *Clostridium aminophilum* DSMZ 10710) and three amylolytic strains (*Prevotella bryantii* B14, *Streptococcus bovis* DSMZ 2048 and *Ruminobacter amylophilus* H-18). All strains were obtained from the Microbiology Unit belonging to the Institut National de la Recherche

Agronomique (INRA), Saint-Gènes-Champanelle, France. All bacteria were cultured for 15 h at 39°C under anaerobic conditions [10].

Antimicrobial assay

Propolis extracts

The assay was conducted in Hungate tubes containing 4 ml of the culture medium [10]. The antimicrobial activity was assessed using three increasing concentrations of the propolis extracts: 250, 500 and 1000 µg ml^{-1}; the control tubes (Con) contained the culture medium and ethanol at 80%. The tubes were inoculated with the strains (5%) and incubated at 39°C for 15 h. The whole procedure was carried out under anaerobic conditions. The bacterial growth was monitored after 15 h using turbidimetry (1 cm cuvette, 600 nm). The assay was conducted in triplicate and mean values are reported.

Phenolic compounds

Different phenolic acids and flavonoids found in the Brazilian propolis were tested. The phenolic acids used were caffeic acid (pure, lot number: 0001416536, Sigma-Aldrich Co., St. Louis, USA), at concentrations 1.5 and 2.0 mg ml^{-1} of culture medium; *p*-coumaric acid (lot number 00003833-KEC, ChromaDex, Irvine, USA), at concentrations 250, 500 and 1000 µg ml^{-1} of culture medium; and Artepillin C ("Artepillin C from propolis", 98%, lot number STN0051, 134 Wako Pure Chemical Industries, Osaka, Japan), at concentrations 20, 40 and 80 µg ml^{-1} of culture medium. The flavonoids tested were chrysin (Chrysin 97%, lot number S36906-269, Sigma-Aldrich Co., St. Louis, USA), at 100, 250, 500 and 1000 µg ml^{-1} of culture medium and naringenin (approximately 95%, lot number 118K1468, Sigma-Aldrich Co., St. Louis, USA), at 33.3 and 53.3 µg ml^{-1} of culture medium. All standards were diluted in ethanol at 80%, filtered and stored protected from light. The control tubes (Con) contained the culture medium and ethanol at 80%. The control assay was conducted in the same manner as for the propolis extracts.

Growth with propolis

The bacterial growth was monitored in Balch tubes containing 8 ml of the culture medium [10] under anaerobic conditions at 39°C for 17 h. The growth of the strains

Prevotella ruminicola 11370, *Fibrobacter succinogenes* S85 and *Clostridium aminophilum* DSMZ 10710 were assessed in the culture medium containing the extracts LLOS B1 and C3, at 250, 500 and 1000 µg of propolis extract ml^{-1}. Control tubes contained the culture medium and ethanol at 80%. Bacterial growth was monitored every 2 hours using turbidimetry (OD 600 nm). The growth assay was conducted in duplicate.

Statistical analysis

Data were subjected to analysis of variance and differences between treatments means were determined by Tukey test. Tests that had *P*-values<0.01 were considered statistically significant.

Results

From the twelve strains tested, nine showed sensitivity to the propolis extracts (Table 1), with greater inhibitory effect observed at the highest concentration of propolis (1000 µg ml^{-1}). Among the fibrolytic species, the strains of *F. succinogenes* and *R. flavefaciens* were more susceptible to the propolis extracts, with great inhibition at 500 µg ml^{-1}. *R. albus* 7 was sensitive to the extracts B1 and C3 at 250 µg ml^{-1}; however, greater inhibition occurred at the concentration 1000 µg ml^{-1}. The antimicrobial activity against *B. fibrisolvens* was observed only at the highest concentration of propolis (1000 µg ml^{-1}) for B1 and C1, with resistance to the C3 extract. The strains of *P. albensis* and *P. ruminicola*, both proteolytic, showed sensitivity to the propolis extracts only at the highest concentration tested; however, the strains of *Peptostreptococcus* sp. and *C. aminophilum*, both hyper-ammonia producing bacteria, showed high sensitivity to all LLOS at the different concentrations, except for the lower concentration (250 µg ml^{-1}) of B1 and C3.

The strain of *S. bovis* was inhibited by the propolis extracts B1 and C1, at 500 µg ml^{-1}, but the extract C3 inhibited *S. bovis* only at the higher concentration (1000 µg ml^{-1}). The strains *R. albus* 20, *P. bryantii* B14 and *R. amylophilus* H-18 were tolerant to all propolis extracts ($P > 0.01$) at all tested concentrations. There was influence of the different extracts (LLOS B1, C1 and C 3) on the antimicrobial activity of propolis.

Table 1 Rumen bacterial strains with sensitivity to the propolis extracts at different concentrations (250, 500 and 1000 µg ml^{-1}) and coefficients of variation (CV)

Extracts[a]	Growth[b]				CV	*P*
	Con	250 µg ml^{-1}	500 µg ml^{-1}	1000 µg ml^{-1}		
	Fibrobacter succinogenes S85					
B1	1.093[a]	1.021[a]	0.037[b]	0.003[b]	5.08	<0.001
C1	1.093[a]	1.072[a]	0.037[b]	0.088[b]	9.04	<0.001
C3	1.093[a]	1.071[a]	0.294[b]	0.000[c]	7.17	<0.001
	Ruminococcus flavefaciens FD-1					
B1	0.773[a]	0.774[a]	0.089[b]	0.057[b]	5.60	<0.001
C1	0.773[a]	0.691[a]	0.081[b]	0.151[b]	14.74	<0.001
C3	0.773[a]	0.762[a]	0.521[b]	0.192[c]	9.23	<0.001
	Ruminococcus albus 7					
B1	1.329[a]	1.157[b]	1.073[b]	0.773[c]	3.30	<0.001
C1	1.329[a]	1.135[ab]	0.842[b]	0.159[c]	8.73	<0.001
C3	1.329[a]	1.142[b]	1.146[b]	0.246[c]	2.19	<0.001
	Butyrivibrio fibrisolvens DSMZ 3071					
B1	1.492[a]	1.416[a]	1.493[a]	0.056[b]	2.75	<0.001
C1	1.492[a]	1.443[a]	1.487[a]	0.109[b]	5.40	<0.000
C3	1.492	1.428	1.481	1.405	3.46	0.206
	Prevotella albensis DSMZ 11370					
B1	1.921[a]	1.680[ab]	1.523[ab]	1.351[b]	6.18	0.002
C1	1.921[a]	1.689[ab]	1.737[a]	1.468[b]	3.49	<0.001
C3	1.921[a]	1.631[ab]	1.674[a]	1.322[b]	5.25	<0.001
	Prevotella ruminicola 11370					
B1	1.872[a]	1.754[a]	1.720[a]	1.255[b]	3.89	<0.000
C1	1.872[a]	1.727[a]	1.646[a]	1.368[b]	3.69	<0.000
C3	1.872[a]	1.735[a]	1.698[ab]	1.381[b]	5.20	0.002
	Peptostreptococcus sp. D1					
B1	0.671[a]	0.430[a]	0.043[b]	0.000[b]	28.66	<0.001
C1	0.671[a]	0.423[b]	0.030[c]	0.000[c]	13.48	<0.001
C3	0.671[a]	0.585[a]	0.057[b]	0.000[b]	19.88	<0.001
	Clostridium aminophilum DSMZ 10710					
B1	0.516[a]	0.482[a]	0.075[b]	0.000[c]	3.88	<0.000
C1	0.516[a]	0.380[b]	0.110[c]	0.009[c]	10.59	<0.000
C3	0.516[a]	0.488[ab]	0.522[a]	0.403[b]	4.43	0.001
	Streptococcus bovis DSMZ 2048					
B1	1.889[a]	1.868[a]	1.698[b]	1.288[c]	1.66	<0.001
C1	1.889[a]	1.849[a]	1.595[b]	0.839[c]	2.20	<0.001
C3	1.889[a]	1.883[a]	1.874[a]	1.564[b]	0.82	<0.001

[a] B1 = 148.13 mg of phenolic compounds g^{-1} of propolis dry extract; C1 = 121.13 mg of phenolic compounds g^{-1} of propolis dry extract; C3 = 70.27 mg of phenolic compounds g^{-1} of propolis dry extract

[b] Values expressed in optical density at 600 nm

Means followed by different letters on the same line are statistically different according to the Tukey test ($P < 0.01$)

The extract with the lowest amount of phenolic compounds (C3 = 70.27 mg g^{-1}) showed lower antimicrobial activity against *C. aminophilum* and *S. bovis* and no activity against the strain of *B. fibrisolvens*.

The antimicrobial activity of some flavonoids (chrysin and naringenin) and phenolic acids (caffeic acid, *p*-coumaric acid and Artepillin C) present in the propolis extracts was evaluated against *R. albus* 7, *B. fibrisolvens*, *F. succinogenes* and *P. albensis*. For flavonoids, naringenin had inhibitory effect against all strains (at 33.3 µg ml^{-1} for *B. fibrisolvens* and *F. succinogenes*. and at 53.3 µg ml^{-1} for *R. albus* 7 and *P. albensis*), while no strain was sensitive to chrysin (at 1000 µg ml^{-1}). For phenolic acids, the caffeic acid showed antimicrobial activity against *R. albus* 7 (at 1500 µg ml^{-1}), and no strain was sensitive to *p*-coumaric acid and Artepillin C (at 1000 µg $ml^{-1}$and 150 µg ml^{-1}, respectively).

Figures 1, 2 and 3 show the effect of the different propolis extracts at the concentrations 250, 500 and 1000 µg ml^{-1} on the growth of *P. ruminicola, C. aminophilum* e *F. succinogenes*. Fig. 1 shows that at the concentration 250 µg ml^{-1} the growth of *P. ruminicola* was not inhibited, however, there was an inhibition on the growth of *C. aminophilum* and *F. succinogenes* with the addition of propolis. The growth of *C. aminophilum* was reduced (P = 0.00574) at 17 h of incubation with the propolis extracts (LLOS B1 = 0.621; LLOS C3 = 0.705 and Control = 1.115), while for *F. succinogenes* both extracts inhibited (P = 0.0008) the growth at 2 h (LLOS C3 = 0.000; LLOS B1 = 0.023 and Control = 0.161) and 10 h (P = 0.00002), with minor bacterial growth for LLOS B1 (0.519), followed by LLOS C3 (0.852) and Control (1.198) treatments.

Fig. 2 shows the growth kinetics at 500 µg of propolis extract ml^{-1}. The extracts did not affect the growth of *P. ruminicola*, however the propolis extracts reduced the growth of *C. aminophilum* and *F. succinogenes*. The LLOS B1 reduced (P = 0.00021) the growth of *C. aminophilum* at 4 h of incubation (0.012), followed by LLOS C3 (0.077) and Control (0.115) treatments. With 6 h, the LLOS B1 affected (P = 0.00710) the growth of *C. aminophilum* (0.173) compared to LLOS C3 (0.224) and Control (0.293); after 17 h of incubation both extracts (LLOS B1 = 0.548 and C3 = 0.622) reduced the bacterial growth (P = 0.00311) when compared to the control treatment (1.115).

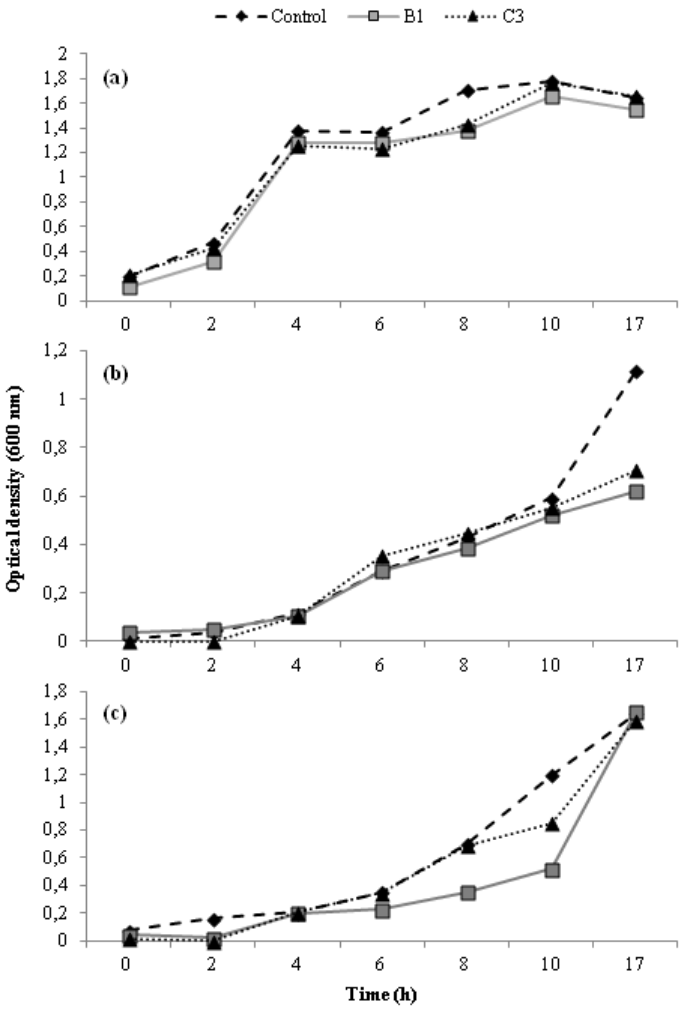

Fig. 1 Growth curves of (a) *P. ruminicola*. (b) *C. aminophilum* and (c) *F. succinogenes* in the presence of propolis extracts B1 (148.13 mg of phenolic compounds g^{-1} of dry extract) and C3 (70.27 mg of phenolic compounds g^{-1} of dry extract) at the concentration 250 µg ml^{-1} of growth medium

The growth of *F. succinogenes* was influenced by the propolis extracts at 2 h (P = 0.00079), 4 h (P = 0.00136) and 17 h (P < 0.00001). At 2 h of incubation, the growth was observed only for control treatment (0.161), and at 4 h a slight increase was observed for LLOS C3 (0.032), and no growth was observed for LLOS B1. At time 17 h, the LLOS C3 inhibited the growth of *F. succinogenes* (0.016), while LLOS B1 showed reduced growth (0.319) when compared to Control (1.64).

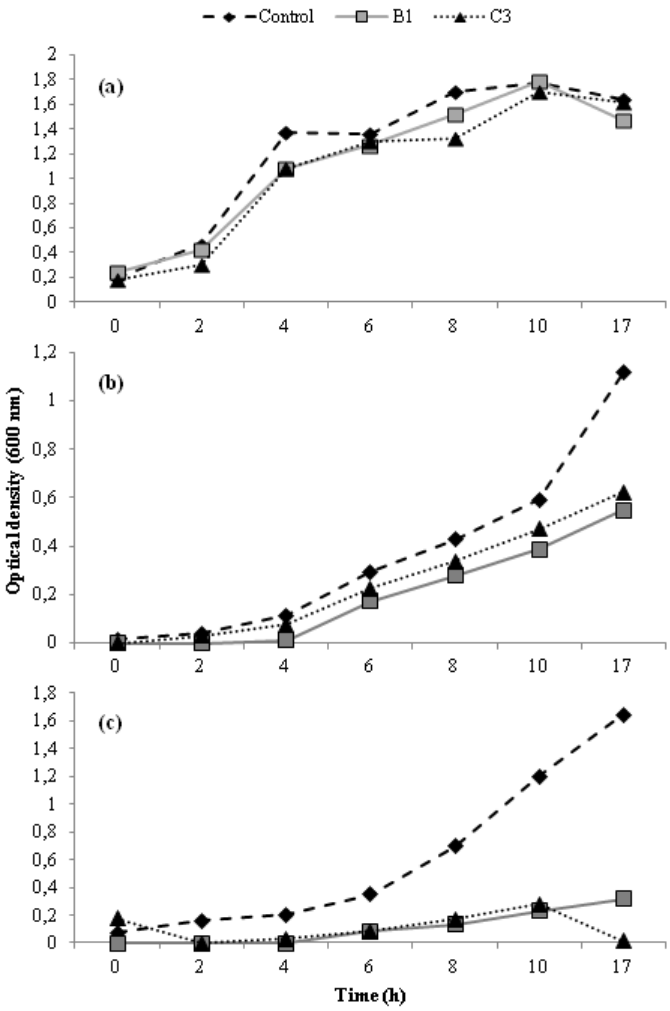

Fig. 2 Growth curves of (a) *P. ruminicola*. (b) *C. aminophilum* and (c) *F. succinogenes* in the presence of propolis extracts B1 (148.13 mg of phenolic compounds g^{-1} of dry extract) and C3 (70.27 mg of phenolic compounds g^{-1} of dry extract) at the concentration 500 µg ml^{-1} of growth medium

The propolis extracts at 1000 µg ml^{-1} decrease ($P < 0.01$) the growth of the studied strains (Fig. 3). The propolis extracts reduced the growth of *P. ruminicola* at 2 h (P = 0.00054) and 4 h (P = 0.00299). The LLOS C3 provided greater reduction (0.154) at 2 h of incubation, when compared with other treatments (LLOS B1 = 0.241 and Control = 0.469). At 4 h, the propolis extracts have drastically reduced the growth of *P. ruminicola* when compared to control (LLOS C3 = 0.387; LLOS B1 = 0.638 and Control = 1.372). There was an effect of propolis on the growth of *C. aminophilum* at 2 h ($P = 0.00276$), 6 h ($P = 0.00563$), 8 h ($P = 0.00637$) and 17 h of

incubation (P = 0.00592). The microbial growth with LLOS B1 was observed only after 8 h; and at 17 h, the B1 showed the lowest growth (0.199) compared to LLOS C3 (0.467) and Control (1.115). The growth of *F. succinogenes* was influenced by the propolis extracts at 2 h (P = 0.00079), 6 h (P = 0.00887), 8 h (P = 0.00138), 10 h (P = 0.00026) and 17 h (P = 0.00070). No growth was observed for extract LLOS B1 in any of the incubation times. The strains of *F. succinogenes* inoculated with LLOS C3 showed some growth after 8 h of incubation, but at 17 h the bacterial growth was completely inhibited.

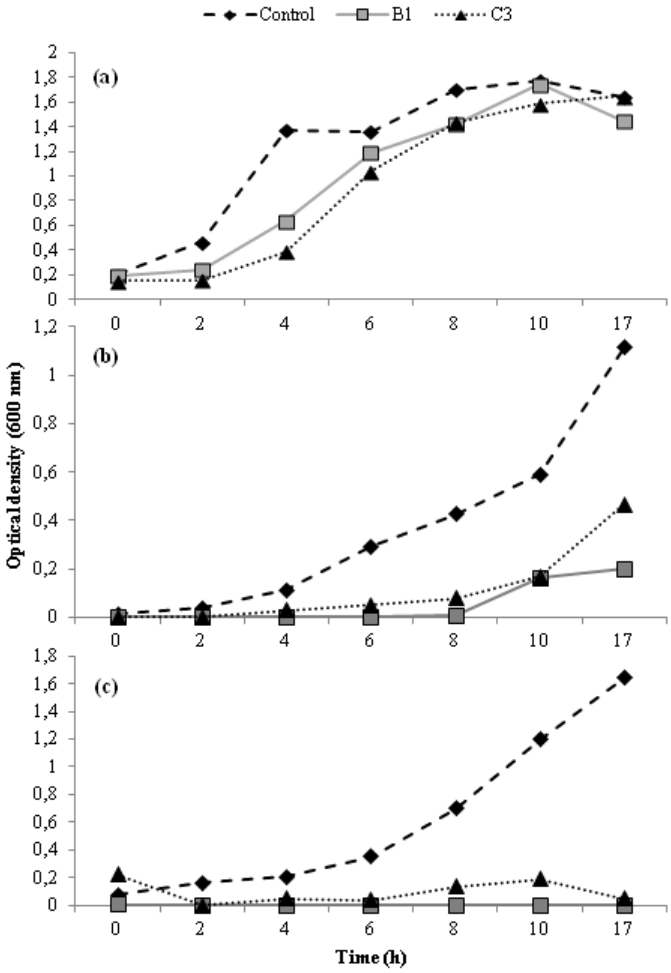

Fig. 3 Growth curves of (a) *P. ruminicola*. (b) *C. aminophilum* and (c) *F. succinogenes* in the presence of propolis extracts LLOS B1 (148.13 mg of phenolic compounds g^{-1} of dry extract) and LLOS C3 (70.27 mg of phenolic compounds g^{-1} of dry extract) at the concentration 1000 µg ml^{-1} of growth medium

Discussion

Many studies show that propolis has antimicrobial activity against *Streptococcus* spp., *Staphylococcus* spp., *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, among others [5, 21, 24, 18]. Although the antimicrobial properties of propolis have been the subject of many investigations, it is difficult to compare the results of different studies, due to the different compositions of propolis and/or different methods used for the evaluation of propolis antibacterial activities [5].

Propolis is considered to be only weakly effective against Gram-negative species [20, 21, 24]; however, in this study, propolis showed strong antimicrobial activity against both Gram-negative and Gram-positive bacteria, as shown in Table 1. The same was observed by Prado et al. [17], that isolated bacteria from the rumen of dairy cows receiving propolis, which were considered as "tolerant to propolis", and found that the Gram-positive bacteria were more resistant to the propolis extracts. Mirzoeva et al. [12] found that propolis had a potent bactericidal effect against Gram-negative *Rhodobacter sphaeroides,* suggesting that the barrier function of the Gram-negative outer membrane is species-dependent, possibly reflecting the porin or lipopolisaccharide composition of the outer membrane. However, in this study, the strain *R. albus* 7 was sensitive to the propolis extracts, while the strain *R. albus* 20 was resistant to all extracts, suggesting that the antimicrobial activity of propolis on same species may differ depending on the strain studied. The strains of *Peptostreptococcus* sp. and *C. aminophilum* (Table 1), both hyper-ammonia producing bacteria, showed high sensitivity to the propolis extracts. Stradiotti Júnior et al. [22] found that propolis was effective in inhibiting the specific activity of ammonia production by rumen microorganisms, both *in vitro* and *in vivo*. Other papers recently published also found the ability of propolis to reduce the ammonia production in the rumen fluid [13, 14]. Further studies correlating the *in vitro* antimicrobial activity of propolis with the effects observed *in vivo* should be performed in order to better understand its action on ruminal metabolism. The different propolis extracts tested (LLOS B1, C1 e C3) affected the antimicrobial activity of propolis. The variation in the chemical composition of propolis render its standardization difficult, and the different solvents used for the extraction (ethanol,

methanol and water) can also extract different compounds influencing, thus, their biological activity [19]. Tosi et al. [23] observed that the solvent employed for the extraction may enhance the potency of the antimicrobial activity of propolis. It is also important to note that, as well as the solvent used, the concentration of propolis used in the extraction of phenolic compounds can influence the chemical composition of the extract. The amount of phenolic compounds present in the LLOS extracts supports this affirmation, since LLOS C3 (highest concentration of propolis and higher alcohol level) showed the lowest amount of phenolic compounds and, therefore, less antimicrobial activity against *C. aminophilum* and *S. bovis* (compared to the other extracts) and no activity against *B. fibrisolvens*.

Among the isolated compounds present in propolis (naringenin, chrysin, caffeic acid, *p*-coumaric acid and Artepillin C), only naringenin inhibited the strains tested. However, the naringenin concentration in the propolis extracts tested is too small, compared to other phenolic compounds. Besides the effect of individual constituents, synergistic effects of several compounds may be responsible for the different pharmacological activities to propolis. Kujumgiev et al. [8] suggested that general biological properties of propolis are due to a natural mixture of its components, and a single propolis constituent does not have an activity greater than that of the total extract.

The growth of the strains of *P. ruminicola*, *C. aminophilum* and *F. succinogenes* was most affected in the highest concentration of propolis (1000 μg ml^{-1}) to all extracts (Fig. 1-3). According to the results obtained in the growth assay, it appears that propolis shows a bacteriostatic effect, except for *F. succinogenes*, in the highest concentration of propolis (Fig. 3). These results are in agreement with Sforcin et al. [20], which found that propolis extracts showed an efficient inhibitory action, however, they cannot be assured of a bactericidal activity, but only a bacteriostatic effect. According to Mirzoeva et al. [12], the bactericidal effect of propolis is due to the presence of an unstable component of propolis. The authors observed an increased lag period of Bacillus *subtilis* growth in the presence of propolis, which seems to be a result of killing of the majority of the bacteria. The resumed growth probably resulted from the growth of surviving cells after inactivation of some very active, but unstable component. The authors suggest that this growth is not due to the selection of resistant forms, but due to the growth of surviving bacteria after the

destruction and/or inactivation of some very potent but biologically labile ingredient of propolis. In conclusion, the different propolis extracts inhibited the growth of most strains tested; in addition, the potential of inhibition was influenced by the extraction conditions of the phenolic compounds present in propolis. Propolis has, thus, antimicrobial activity against both Gram-positive and Gram-negative bacteria, and against various species. The extract B1 and C1 showed stronger inhibition than LLOS C3, which is the extract with the lowest amount of phenolic compounds. The study of the biological properties of propolis should be linked to a detailed investigation of its chemical composition, to better understand its effects. The mechanism of action of propolis against individual rumen bacteria as well as its effect on the balance of the rumen microbial communities remains to be elucidated.

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CONSIDERAÇÕES FINAIS

As diferentes condições de extração alteram a composição fenólica dos extratos de própolis, influenciando sua composição química;

Sob diferentes condições de extração, alguns flavonoides e ácidos fenólicos podem não ser extraídos, interferindo na caracterização fenólica dos extratos de própolis;

No trabalho realizado *in vivo*, os produtos à base de própolis (LLOS) interferiram no metabolismo ruminal, sendo que o LLOS C1 teve efeito positivo no metabolismo de nitrogênio, pois reduziu sua perda na forma de amônia;

Os diferentes LLOS não afetaram a produção e qualidade do leite, entretanto, alteraram a composição em ácidos graxos e aumentaram a capacidade antioxidante, a qual foi maior para os LLOS com maior quantidade de compostos fenólicos;

No trabalho realizado *in vitro*, os diferentes extratos de própolis apresentaram ação antimicrobiana tanto contra bactérias Gram-positivas quanto Gram-negativas, assim como contra várias espécies. A condição de extração influenciou a atividade antimicrobiana, uma vez que os LLOS B1 e C1 apresentaram maior poder inibitório do que o C3, o qual contém menor quantidade de compostos fenólicos;

O estudo das propriedades biológicas da própolis deve ser ligado a uma investigação detalhada da sua composição química, para melhor compreender os seus efeitos. O mecanismo de ação da própolis contra bactérias ruminais individuais, bem como seu efeito sobre o equilíbrio das comunidades microbianas do rúmen, permanece a ser elucidado.