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Effects of phenolic compounds in propolis on digestive and ruminal parameters in dairy cows

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ABSTRACT - Four rumen-cannulated primiparous lactating cows were studied in a 4 × 4 Latin square design experiment to evaluate the effects of propolis-based products (PBP) with different concentrations of propolis and alcohol levels on total digestibility, (TD), ruminal digestibility (RD), intestinal digestibility (ID), pH, ruminal ammonia-nitrogen production (NH_3-N), rumen microbial synthesis, and blood parameters. The feed consisted of 591.9 g/kg corn silage and 408.1 g/kg concentrate (dry matter [DM] basis), and treatments differed with regard to the inclusion (via ruminal cannula) or exclusion of PBP as follows: control (without the PBP), PBP B1 (3.81 mg of phenolic compounds/kg of ingested DM), PBP C1 (3.27 mg of phenolic compounds/kg of ingested DM), and PBP C3 (1.93 mg of phenolic compounds/kg of ingested DM). Inclusion of PBP reduced the RD of dietary crude protein (CP). Treatment PBP C1 reduced ruminal NH_3-N production, while PBP B1 increased the ID of CP relative to that in the control. These findings indicate that propolis had a positive effect on rumen nitrogen metabolism. Rumen pH, efficiency of microbial protein synthesis, and blood parameters were not affected by addition of PBP, but there were significant effects on the other parameters when the treatments containing propolis were contrasted. Higher TD of DM (0.717 vs. 0.685), OM (0.737 vs. 0.703), and CP (0.760 vs. 0.739), as well as higher NDF (0.622 vs. 0.558) and TDN (0.747 vs. 0.712) were observed when comparing PBP C1 with C3. Inclusion of propolis in diets for dairy cows have positive effects on protein metabolism in the rumen. Variation in the amounts of phenolic compounds in the different PBP may explain the diverse effects on the digestive parameters evaluated.

Key Words: additive, ammonia nitrogen, flavonoids, phenolic acids, ruminants

Introduction

The commonly used additives in ruminant nutrition have an important role as modulators of the end products of rumen fermentation; however, the use of antibiotics in animal feeds is facing decreased social acceptance, and their use has been forbidden in the European Union since January 2006 (Calsamiglia et al., 2007). For this reason, there is substantial interest in evaluating the potential of natural antimicrobials such as plant extracts to modify rumen microbial fermentation (Busquet et al., 2006). The plants of interest produce a diverse array of secondary compounds, which have benefits in ruminant production (Wallace, 2004). These compounds, including phenylpropanoids and flavonoids, are known to have an impact on rumen microbial metabolism by inducing changes in the fermentation conditions (pH, propionate proportion, and protein degradation) (Broudiscou et al., 2002; Balcells et al., 2012).

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Propolis is a resinous material collected by bees from plant buds and exudates; flavonoids are considered the main class of biologically active phenolic compounds in propolis (Castro et al., 2007). Other compounds are also involved, such as cinnamic acid derivatives and Artepillin C (3, 5-diprenyl-4-hydroxycinnamic acid), one of the major phenolic acids found in Brazilian propolis (Estrada et al., 2008). These phenolic compounds are responsible for the antimicrobial and antioxidant activities of propolis (Santos Neto et al., 2009; Frozza et al., 2013).

However, in order to be officially accepted by major health agencies, propolis requires a chemical standardization to ensure its quality, safety and efficacy (Bankova, 2005); its composition is closely related to the ecology of the flora of each region visited by bees (Park et al., 2002). Furthermore, the concentration of propolis and the alcohol level used to extract the active substances may influence the chemical composition of the propolis extract (Cottica et al., 2011).

In studies involving ruminant metabolism and nutrition, propolis reduced ammonia (NH_3) production *in vitro* (Ozturk et al., 2010) and the number of ruminal ciliate protozoa in buffaloes (Rísposli et al., 2009). Therefore, we evaluated the effects of phenolic compounds from three

propolis-based products with different concentrations of propolis and levels of alcohol on feed intake, digestibility (ruminal and intestinal) and blood parameters in lactating dairy cows.

Material and Methods

Four primiparous Holstein cows, 147 days into their lactating period, with a mean body weight (BW) of 550±34.16 kg, were selected. The cows were cannulated in the rumen, housed in tie stalls, and subjected to two daily milkings (06.00 h and 15.00 h). The animals were randomly assigned to a 4 × 4 Latin square, with four periods and four treatments, and were treated according to the guidelines of the Committee for Ethical Care and Use of Animals in Research of Universidade Estadual de Maringá (No. 027/2011).

The propolis-based products used in the treatments 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) of alcohol] and were prepared according to Franco and Bueno (1999). Propolis samples were obtained from the apiary of the Experimental Farm of Iguatemi (FEI), belonging to Universidade Estadual de Maringá, Paraná State, Brazil. The apiary is located within a reserve of eucalyptus (*Eucalyptus* sp.) plants surrounded by native forest and characterized by the presence of alecrim-do-campo (*Baccharis dracunculifolia*) plants. Propolis-based products (PBP) are protected under the intellectual property right No. 0605768-3 in Brazil. Propolis extracts were obtained by turbo extraction for 15 min. The extracts were filtered under vacuum, after which the alcohol was removed in a rotary evaporator (Buchi, model RT 210). Subsequently, the extracts were spray-dried in a nebulizer (MSD 1.0, Labmaq, Ribeirão Preto, SP, Brazil) with a capacity of 1 L/h and an inlet temperature of 100 °C. The powder of PBP fed to the animals contained this dried propolis extract and an excipient (ground corn and soybean meal). The excipient was used to add volume to the propolis extract and facilitate feeding. The propolis-based products differed both as to the chemical composition and amount of phenolic compounds, resulting in three unique products identified (Table 1).

These compounds were quantified using high-performance liquid chromatography with a photodiode array detector (Alliance HPLC-PDA, Waters Co., Milford, MA, USA).

The experimental diet (Table 2) consisted of 591.9 g/kg corn silage and 408.1 g/kg concentrate (DM basis), and differed with the inclusion or non-inclusion of the PBP, resulting in four treatments: control (without phenolic compounds from the PBP), PBP B1 (3.81 mg of phenolic compounds/kg of ingested DM), PBP C1 (3.27 mg of phenolic compounds/kg of ingested DM), and PBP C3 (1.93 mg of phenolic compounds/kg of ingested DM).

Table 1 - Flavonoids and phenolic acids identified in the propolis-based products (PBP)[1,2]

	PBP B1	PBP C1	PBP C3
	mg/g of dry extract		
Chlorogenic acid	n.d.	0.31	n.d.
Caffeic acid	5.27	6.17	4.03
p-coumaric acid	9.30	10.59	6.85
Benzoic acid	0.76	1.56	0.58
CAPE	3.54	3.48	1.93
Artepillin C	9.86	9.45	6.01
Apigenin	9.95	7.39	4.83
Pinocembrin	6.39	4.70	3.02
Galangin	1.93	n.d.	n.d.
Chrysin	5.07	3.44	2.09
Acacetin	5.27	4.74	2.65

n.d. - not detected.
CAPE - caffeic acid phenethyl ester
[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] PBP B1 = 3.81 mg of phenolic compounds/kg of IDM; PBP C1 = 3.27 mg of phenolic compounds/kg of IDM; PBP C3 = 1.93 mg of phenolic compounds/kg of IDM.

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

	g/kg									Diet (%)
	DM	OP	CP	EE	NDF	ADF	TC	NFC	TDN	
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. premix[1]	990.0	-	-	-	-	-	-	-	-	1.98
Limestone	991.4	-	-	-	-	-	-	-	-	0.32
Ammonium sulfate	990.0	-	1,250	-	-	-	-	-	-	0.14
Diet	539.4	934.1	160.6	52.60	451.8	236.9	722.4	270.6	714.9	100.0

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.
[1] Composition of the vitamin and mineral premix (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 vitamin A; 78,000 IU of vitamin D3; 450 IU of vitamin E.

The experimental diet was formulated according to the recommendations of the National Research Council (NRC, 2001) for lactating cows with body weights (BW) of approximately 550 kg, at 21 weeks in lactation, and with an estimated milk yield of 25.0 kg/day (with a 3.8% fat content). Net energy for lactation (NE_L) was estimated using the following equation: NE_L (Mcal/kg) = 0.0245 × %Total digestible nutrients – 0.12 (NRC, 2001), thus generating a value of 1.63 Mcal/kg.

The propolis-based products were inserted in the rumen via a ruminal cannula at the time of feeding, in the form of two daily doses adjusted to 15 g of the excipient (7.5 g of PBP at 08.00 h and 7.5 g at 16.00 h). Feed intake was recorded daily and the amounts offered were adjusted to allow for 100 g/kg refusals as fed. Cows were weighed at the beginning and end of each experimental period.

The study consisted of four experimental periods of 24 days each (14 days for adaptation, 7 days for sample collection and 3 days for washout). From the second to the fifth day of the collection period, feces and omasal digesta were sampled. Fecal samples (100 g) were collected directly from the rectum, while omasal digesta samples (400 mL) were collected by suction of the omasal contents, according to the technique described by Leão et al. (2005). On the first day, the collection was performed at 20.00 h, on the second day at 16.00 h, on the third day at 12.00 h and on the fourth day at 08.00 h, totaling four samples (feces and digesta) per animal in each period. After the collection period, samples of feed, feces and digesta were dried in a ventilated oven (55 °C for 72 h), ground to a particle size of 1 mm, and mixed in equal quantities to form composite samples. Daily feed intake was estimated as the difference between the supplied feed and refusals in the trough. During the experimental period, samples of feed and refusals were collected and a representative composite sample was drafted per animal in each treatment.

In the last two days of each experimental period, the ruminal fluid was collected via a ruminal cannula to determine pH and ammonia nitrogen (NH_3-N). Urine was collected to determine the efficiency of microbial protein synthesis. The collection began before the first feeding (08.00 h), which was set as time zero (0) and 2, 4, 6 and 8 h post-feeding, with five samples obtained per animal per period. To determine the NH_3-N, the material was filtered to obtain 50 mL of ruminal fluid. Immediately after collection, the pH was determined and 1 mL of sulfuric acid (H_2SO_4) 1:1 was added to the sample. To determine the daily flows of DM and digesta, chromium oxide (Cr_2O_3) was used as an external marker. Two intra-ruminal doses (5.0 g) for a total of 10.0 g Cr_2O_3/day, which was previously weighed in a hygroscopic paper, were provided daily (at 08.00 h and 16.00 h). NH_3-N was determined according to Vieira (1980).

Digestive parameters were estimated according to the equations described by Coelho da Silva and Leão (1979). Procedures to determine DM (method no. 934.01), organic matter (OM) (determined by ash content, method no. 924.05), crude protein (CP, method no. 920.87), and to prepare ether extracts (EE, method no. 920.85) were conducted according to AOAC (1990). Neutral detergent fiber (NDF) was analyzed with the procedure of Van Soest et al. (1991) with amylase and sodium sulfite. Acid detergent fiber (ADF) was determined according to method no. 973.18 (AOAC, 1990). Total carbohydrates (TC) were obtained 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). The TDN content of the experimental diets was calculated using the following equation: %TDN = %DCP + %DNDF + %DNFC + % (DEE × 2.25), in which DCP = digestible crude protein, DNDF = digestible neutral detergent fiber, DNFC = digestible non-fiber carbohydrates, DEE = digestible 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 0.036 N sulfuric acid (H_2SO_4) 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 cycles, which were combined into a composite sample. A 10 mL aliquot of milk was mixed with 5 mL of 25% trichloroacetic acid ($C_2HCl_3O_2$), filtered, and stored at 5 °C for subsequent analysis of urea and allantoin.

Allantoin was analyzed using the method described by Chen and Gomes (1992). To determine creatinine, uric acid, and urea, analyses were performed using commercial kits (Labtest). From the concentration of creatinine in the spot urine sample, the urinary volume was estimated (L) by dividing the daily excretion of creatinine (mg/kg BW) by creatinine concentration (mg/L). For the determination of daily creatinine excretion per kg of BW, the average value of 23.41 mg/kg of BW was used as discussed by Oliveira et al. (2001), who determined the creatinine excretions of Holstein cows fed diets comprising 60:40 forage-to-concentrate ratio; these characteristics are similar to those in our study. The microbial nitrogen (N) production was calculated from the amount of absorbed purines, which was

estimated as the sum of the excretion of purine derivatives (PD) in milk and urine, after which the synthesis of microbial N compounds in the rumen was calculated based on the absorbed purine, according to Chen and Gomes (1992). Microbial protein synthesis (MPS) was estimated by multiplying the microbial N synthesis by 6.25, while the efficiency of microbial protein synthesis (EMPS) was determined as follows: EMPS (g/kg) = MPS (g)/TDNI (kg), in which TDNI = total digestible nutrients intake.

To determine blood urea, blood samples were collected on day 21 of each experimental period, 4 h after the first feeding. Immediately after sampling, the tubes (containing heparin) were centrifuged at 2500 rpm for 15 min to obtain the plasma. The centrifuged samples were transferred to labeled plastic tubes, stored in an insulated box, and immediately transported to a laboratory, where analysis was performed using a commercial kit in an automatic analyzer for blood biochemistry (Vitalab Selectra, Merck & Co. Inc., Whitehouse Station, NJ, USA).

Data were interpreted with analysis of variance by using the GLM procedure of Statistical Analysis System (SAS, version 8.01). The mathematical model used for the analysis was as follows: $Y_{ijk} = \mu + A_i + P_j + T_k + e_{ijk}$, in which Y_{ijk} = observed variables, μ = overall mean, A_i = effect of animal i, ranging from 1 to 4; P_j = effect of period j varying from 1 to 4; T_k = k effect of the treatment, ranging from 1 to 4; and e_{ijk} = random error. Statistical analyses of ruminal parameters (pH and NH_3-N) were performed using a split-plot design, with treatments as plots and collection times as subplots. Differences between treatment means were determined using Tukey's HSD test. Values with $\alpha = 0.05$ were considered statistically significant.

Results

The amount of PBP supplied to the cows resulted in a variation in the intake of flavonoids and phenolic acids per kg of ingested DM (Table 3). The PBP C3 had the lowest concentration of total flavonoids and phenolic acids, resulting in a lower intake of these compounds.

No effect (P>0.05) on DM intake and nutrients was observed between treatments, but a high NDF intake (P = 0.009) was observed in the PBP B1 treatment (Table 4). The inclusion of PBP in the diet affected (P<0.05) the ruminal digestibility of DM, OM, CP, and TC, with lower ruminal digestibility of these nutrients in the PBP C1 treatment compared with that in the control. Likewise, the inclusion of PBP had significant effects (P<0.05) on the intestinal digestibility of DM, OM, CP, and TC (Table 4). For CP, greater intestinal digestibility (P<0.05) was observed when PBP B1 was present compared with control. The diets containing PBP B1 and C1 presented higher intestinal digestibility of DM, OM, and TC (P<0.05) than control diet.

The different PBP did not affect DM, OM, CP, NDF, and TDN (P>0.05) (Table 5) compared with control diet. Among the propolis-based products, PBP B1 was responsible for greater total digestibility of DM, OM, NDF, ADF and TC (P<0.05) compared with PBP C3, but did not differ (P>0.05) from those in the PBP C1.

Inclusion of PBP in the diet had no effect (P>0.05) in terms of the interaction between treatment and collection time for rumen pH (Figure 1). Rumen pH exhibited a quadratic curve as a function of time in terms of hours post-feeding ($pH = 6.86944 - 0.42107X + 0.042898X^2$, $r^2 = 0.736\%$), with an estimated minimum pH of 5.83 at 4.9 h.

No interaction was observed between treatment and collection time (P>0.05) for NH_3-N concentration in the rumen fluid (Figure 2); however, the mean concentrations of NH_3-N in the rumen were influenced (P<0.05) by including PBP C1 in the diet (Table 6).

The behavior of NH_3-N as a function of time after feeding was quadratic, in which $NH_3\text{-N} = 16.4810 + 7.96253X - 0.871208X^2$, with $r^2 = 0.951\%$. The highest estimated concentration of NH_3-N in the ruminal fluid was 34.67 mg/dL at 4.6 h after feeding and the minimum concentration was 16.48 mg/dL at 0 h.

The propolis-based products had no effect (P>0.05) on the excretion of purine derivatives in urine and milk,

Table 3 - Total flavonoids and phenolic acids quantified in the propolis-based products (PBP) supplied daily to dairy cows

Compounds	Propolis-based product[1,2] (mg/kg IDM)			P-value	SEM
	PBP B1	PBP C1	PBP C3		
Total flavonoids	2.81a	2.14b	1.22c	<0.001	0.028
Artepillin C and CAPE	0.40a	0.41a	0.25b	<0.001	0.006
Total phenolic acids[3]	1.00b	1.13a	0.71c	<0.001	0.017

Different letters in the same row are statistically different (P<0.05) by Tukey's test.
IDM - ingested dry matter; SEM - standard error of the mean.
[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] PBP B1 = 3.81 mg of phenolic compounds/kg of IDM; PBP C1 = 3.27 mg of phenolic compounds/kg of IDM; PBP C3 = 1.93 mg of phenolic compounds/kg of IDM.
[3] Sum of the phenolic acids grouped at the beginning of the chromatogram with CAPE and Artepillin C.

Table 4 - Feed intake, ruminal digestibility[1] and intestinal digestibility[2] of DM and nutrients in dairy cows fed a diet with (PBP) or without (CON) addition of phenolic compounds from propolis

Parameters	Treatments[3]				SEM	P-value
	CON	PBP B1	PBP C1	PBP C3		
			Dry matter			
Intake (kg/day)	15.66	16.34	15.60	15.31	0.212	0.064
Omasal flow (kg/day)	7.62	8.11	8.04	7.44	0.178	>0.100
Ruminal digestibility (kg/kg)	0.513a	0.503ab	0.483b	0.514a	0.010	0.022
Fecal flow (kg/day)	4.87	4.61	4.64	4.82	0.150	0.197
Intestinal digestibility (kg/kg)	0.362b	0.430a	0.422a	0.352b	0.021	0.004
			Organic matter			
Intake (kg/day)	14.60	15.23	14.55	14.28	0.194	0.062
Omasal flow (kg/day)	6.46ab	6.84a	6.80ab	6.26b	0.136	0.063
Ruminal digestibility (kg/kg)	0.557a	0.550a	0.532b	0.561a	0.009	0.013
Fecal flow (kg/day)	4.25	4.00	4.05	4.23	0.135	0.125
Intestinal digestibility (kg/kg)	0.343b	0.414a	0.404a	0.323b	0.024	0.008
			Crude protein			
Intake (kg/day)	2.51	2.67	2.52	2.46	0.047	0.079
Omasal flow (kg/day)	1.87b	2.16a	2.05ab	1.99ab	0.055	0.048
Ruminal digestibility (kg/kg)	0.256a	0.189b	0.182b	0.190b	0.023	0.007
Fecal flow (kg/day)	0.63	0.64	0.69	0.64	0.019	0.214
Intestinal digestibility (kg/kg)	0.657b	0.700a	0.663b	0.678ab	0.015	0.030
			Neutral detergent fiber			
Intake (kg/day)	6.84b	7.15a	6.81b	6.61b	0.070	0.009
Omasal flow (kg/day)	2.97ab	3.20a	3.23a	2.88b	0.071	0.031
Ruminal digestibility (kg/kg)	0.565	0.552	0.525	0.563	0.015	0.113
Fecal flow (kg/day)	2.92	2.70	2.74	2.92	0.106	0.375
Intestinal digestibility (kg/kg)	0.018ab	0.158a	0.152a	0.001b	0.037	0.037
			Total carbohydrates			
Intake (kg/day)	11.26	11.68	11.19	11.00	0.137	0.059
Omasal flow (kg/day)	4.05ab	4.37a	4.30a	3.87b	0.100	0.041
Ruminal digestibility (kg/kg)	0.639a	0.625ab	0.615b	0.648a	0.011	0.027
Fecal flow (kg/day)	3.54	3.28	3.29	3.52	0.131	0.396
Intestinal digestibility (kg/kg)	0.126b	0.247a	0.235a	0.089b	0.044	0.009

Different letters in the same row are statistically different (P<0.05) by Tukey's test.
SEM - standard error of the mean; IDM - ingested dry matter.
[1] Based on the amount ingested.
[2] Based on the amount that reached the duodenum.
[3] PBP B1 = 3.81 mg of phenolic compounds/kg of IDM; PBP C1 = 3.27 mg of phenolic compounds/kg of IDM; PBP C3 = 1.93 mg of phenolic compounds/kg of IDM.

Table 5 - Total digestibility of nutrients and total digestible nutrients in dairy cows fed diets with (PBP) or without (CON) addition of phenolic compounds from propolis

Parameters	Treatments[1] (kg/kg)				SEM	P-value
	CON	PBP B1	PBP C1	PBP C3		
DM total digestibility	0.689ab	0.717a	0.702ab	0.685b	0.008	0.034
OM total digestibility	0.709ab	0.737a	0.721ab	0.703b	0.008	0.030
CP total digestibility	0.745ab	0.760a	0.725b	0.739ab	0.009	0.022
EE total digestibility	0.912	0.920	0.916	0.908	0.008	0.385
NDF total digestibility	0.573ab	0.622a	0.598ab	0.558b	0.016	0.025
ADF total digestibility	0.552	0.591	0.570	0.526	0.018	0.054
TC total digestibility	0.685	0.718	0.706	0.680	0.011	0.053
NFC total digestibility	0.860	0.870	0.881	0.867	0.008	0.316
Total digestible nutrients	0.719ab	0.747a	0.732ab	0.712b	0.008	0.022

Different letters in the same row are statistically different (P<0.05) by Tukey's test.
SEM - standard error of the mean; IDM - ingested dry matter.
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.
[1] PBP B1 = 3.81 mg of phenolic compounds/kg of IDM, PBP C1 = 3.27 mg of phenolic compounds/kg of IDM, PBP C3 = 1.93 mg of phenolic compounds/kg of IDM.

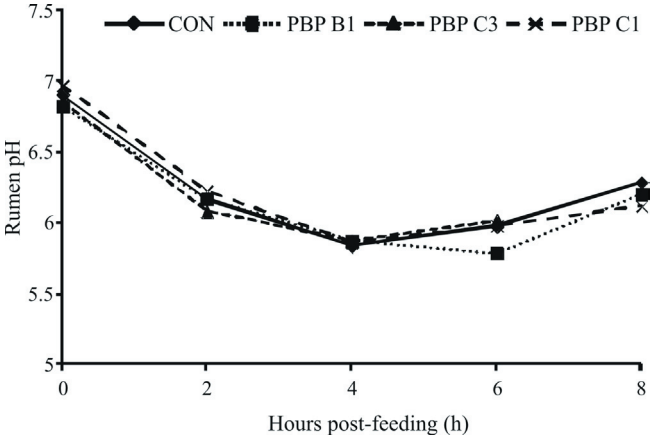

Figure 1 - Ruminal pH as affected by hours post-feeding in dairy cows fed diets with (PBP) or without (CON) addition of phenolic compounds from propolis.

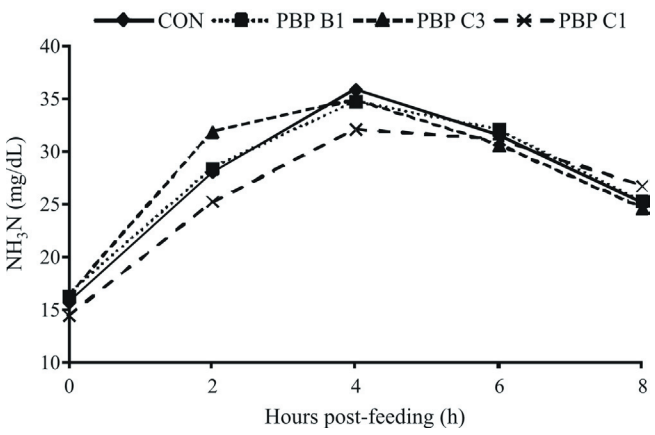

Figure 2 - NH_3-N concentration in the rumen fluid as affected by hours post-feeding in dairy cows fed diets with (PBP) or without (CON) addition of phenolic compounds from propolis.

Table 6 - Ruminal pH and ammonia nitrogen (NH_3-N) of dairy cows fed diets with (PBP) or without (CON) addition of phenolic compounds from propolis

Parameters	Treatments[1]				P-value	SEM
	CON	PBP B1	PBP C1	PBP C3		
pH	6.24	6.17	6.22	6.23	0.158	0.053
N-NH_3 (mg/dL)	27.27a	27.37a	25.94b	27.63a	0.002	0.183

Different letters in the same row are statistically different (P<0.05) by Tukey's test.
SEM -standard error of the mean; IDM - ingested dry matter.
[1] PBP B1 = 3.81 mg of phenolic compounds/kg of IDM; PBP C1 = 3.27 mg of phenolic compounds/kg of IDM; PBP C3 = 1.93 mg of phenolic compounds/kg of IDM.

Table 7 - Urinary volume, urinary excretion of purine derivates, microbial protein synthesis and efficiency of microbial protein synthesis of dairy cows fed diets with (PBP) or without (CON) addition of phenolic compounds from propolis

Parameters	Treatments[1]				SEM
	CON	PBP B1	PBP C1	PBP C3	
Urinary volume (L/day)	16.85	16.57	18.12	19.38	1.48
			Purine derivatives (PD)		
Allantoin (mmol/day)	232.33	215.82	274.16	240.82	32.51
Uric acid (mmol/day)	24.71	24.20	26.70	30.22	1.57
Milk PD (mmol/day)	16.12	16.51	17.23	16.36	0.30
Purine derivatives (mmol/day)	273.16	256.52	318.09	287.39	34.11
Allantoin (% of total PD)	84.67	83.78	85.97	83.15	1.06
Uric acid (% of total PD)	9.25	9.59	8.47	10.73	0.49
Milk PD (% of total PD)	6.08	6.63	5.56	6.12	0.58
			Absorbed microbial purines		
mmol/day	270.66	251.57	323.81	287.64	40.11
			Microbial nitrogen compounds		
g/day	196.77	182.89	235.41	209.11	29.16
			Microbial protein synthesis (MPS)		
g/day	1229.81	1143.06	1471.30	1306.99	182.28
			Efficiency of microbial protein synthesis		
g MPS/kg of total digestible nutrients	111.52	104.56	126.76	116.71	16.74

SEM - standard error of the mean; IDM - ingested dry matter.
[1] PBP B1 = 3.81 mg of phenolic compounds/kg of IDM; PBP C1 = 3.27 mg of phenolic compounds/kg of IDM; PBP C3 = 1.93 mg of phenolic compounds/kg of IDM.

on microbial protein synthesis (g/day), or on microbial efficiency (g MPS/kg of TDN) (Table 7). Likewise, the efficiency of microbial synthesis was not affected (P>0.05) by inclusion of PBP in the diet.

The addition of PBP did not change N utilization (Table 8), which averaged 18.72, 14.63, and 874.12 mg/dL, respectively, for blood urea N (BUN), milk urea N (MUN), and urine urea N (UUN).

Table 8 - Mean concentrations of urea and urea nitrogen in the blood, milk and urine of dairy cows fed diets with (PBP) or without (CON) addition of phenolic compounds from propolis

Parameters	Treatments[1] (kg/kg)				SEM	P-value
	CON	PBP B1	PBP C1	PBP C3		
Blood urea mg/dL	38.50	42.75	40.75	38.25	1.42	0.186
Milk urea mg/dL	31.40	33.42	31.26	29.16	1.65	0.415
Urine urea mg/dL	1790.0	1832.5	2000.0	1860.0	188.83	0.769
Blood urea nitrogen mg/dL	17.99	19.98	19.04	17.87	0.66	0.186
Milk urea nitrogen mg/dL	14.68	15.62	14.61	13.63	0.77	0.415
Urine urea nitrogen mg/dL	836.45	856.31	934.58	869.16	88.24	0.769
Milk protein (%)	3.76	3.77	3.63	3.61	0.11	0.980

SEM - standard error of the mean; IDM - ingested dry matter.
[1] PBP B1 = 3.81 mg of phenolic compounds/kg of IDM; PBP C1 = 3.27 mg of phenolic compounds/kg of IDM; PBP C3 = 1.93 mg of phenolic compounds/kg of IDM.

Discussion

In general, PBP do not appear to affect feed intake when propolis is supplied in the form of powder, as verified in feedlot cattle diets (Valero, 2010; Simioni, 2011; Zawadzki et al., 2011; Aguiar et al., 2012) and forage-based diets (Prado et al., 2010). However, Loureiro et al. (2007) found a reduction in DM intake in lambs fed diets containing 15 and 30 mg of propolis extract/kg of BW as compared with control. In all likelihood, the forms in which propolis is included in the diet (powder, liquid, or directly in the rumen) and dosages, together with the type of diet and animal, are responsible for the diverse effects on DM intake.

In our study, it was observed that the three PBP reduced the ruminal digestibility of CP (P = 0.007), corroborating the findings of Prado et al. (2010). Similarly, Simioni (2011) observed lower ruminal digestibility of CP in cows fed PBP C1 (P<0.05) compared with those fed monensin, but this was not significantly different from that in the control. These data suggest that PBP enhance N metabolism in the rumen, by reducing the populations of NH_3-producing bacteria, and therefore, increasing the flow of microbial protein to the intestine (P = 0.048). Furthermore, PBP B1 improved the intestinal digestibility of CP compared with control, which is beneficial to the animal, because maximizing the capture of degradable N not only improves the supply of amino acids to the small intestine but also decreases N losses (Bach et al., 2005). Furthermore, Aguiar et al. (2013) found that the propolis extracts B1 and C1 inhibited the growth of the hyper-ammonia-producing bacteria *Peptostreptococcus* sp. D1 and *Clostridium aminophilum in vitro*. Thus, PBP may act on the main ammonia-producing bacteria, reducing energy loss and increasing the flow of microbial protein to the intestine, where these proteins are absorbed.

The inclusion of PBP in the diet inhibited ruminal digestibility and changed the primary site of digestion (rumen) for most nutritional components, which was reflected in significant increases in the intestinal digestibility of DM, OM, and TC for PBP B1 and C1 compared with control.

The chemical composition and amount of phenolic compounds in the PBP influenced the total digestibility of DM, OM, CP, NDF, and TDN (Table 5). However, Aguiar et al. (2012) reported no effects of PBP C1 on the total digestibility of DM, OM, NDF, TC, and TDN compared with control treatment. It is noteworthy that the PBP dosage used by Aguiar et al. (2012) was two times lower than that used in our study. In this study, the higher total digestibility of DM and OM linked to the inclusion of PBP B1 rather than PBP C3 could be attributed to the daily supplemented amounts of flavonoids (PBP B1 = 2.81 mg/kg of IDM vs. PBP C3 = 1.22 mg/kg of IDM) and phenolic acids (PBP B1 = 1.00 mg/kg of IDM vs. PBP C3 = 0.71 mg/kg of IDM).

New evidence involving the mechanisms behind the antimicrobial activity of flavonoids has been discovered: inhibition of cell wall and cell membrane synthesis (Cushnie and Lamb, 2011). Accordingly, a single flavonoid may have different mechanisms of action; in propolis, the synergy between phenolic compounds may hinder the understanding of the antimicrobial activity of a particular compound. Among the phenolic acids, the presence of caffeic acid phenethyl ester (CAPE) and Artepillin C was noteworthy. Both the compounds possess strong antimicrobial activity, but their mechanisms of action have not been fully elucidated (Estrada et al., 2008; Bankova, 2009).

The observation that the total digestibility of CP was higher in the PBP B1 than in the PBP C1 treatment (P<0.05) was in contrast with the results of previous studies on PBP, which detected no significant differences between various extracts and/or dosages of PBP (Valero, 2010; Simioni, 2011;

Aguiar et al., 2012). However, effects on the ruminal metabolism of CP as well as an increase in microbial protein flow to the intestine were observed (Prado et al., 2010).

Effects of PBP on the total digestibility of EE have been shown, although this was not observed in the present study. Prado et al. (2010) revealed that the total digestibility of EE in cattle was reduced by adding PBP C1 compared with the control diet and monensin. However, these results contradict those found by Valero (2010), indicating a higher total digestibility of EE with PBP C1 compared with control diet and monensin. Furthermore, Simioni (2011) reported a tendency (P = 0.08) of PBP C1 to increase the total digestibility of EE. However, it is important to consider the forage-to-concentrate ratio supplied to the animals and the dosages of PBP, both of which varied among the cited experiments. Prado et al. (2010) supplied forage-based diets, whereas Valero (2010) and Simioni (2011) used highly concentrate diets. 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 hydrolyze galacto- and phospholipids, other lipolytic species would be expected to predominate in grazing animals. An example is *Butyrivibrio* spp., which hydrolyzes phospho- and galactolipids but does 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, with consequent effects on lipid digestion.

The PBP B1 treatment improved the total digestibility of DM, OM, NDF, ADF and TC ($P<0.05$) compared with PBP C3, but did not differ ($P>0.05$) from PBP C1. According to Prado et al. (2010), the observed differences among the PBP may be related not only to the concentration of propolis, but also to the concentrations of alcohol used in the extraction solvent. The authors concluded that higher alcohol concentrations might facilitate the solubilization of resin and wax present in propolis, which induces the release of phenolic compounds. This hypothesis is reinforced by the results obtained in our study, where PBP C3 showed lower concentrations of flavonoids and phenolic acids than PBP B1 and C1.

The propolis-based products did not affect rumen pH. However, Simioni (2011) found that rumen pH remained higher ($P<0.05$) in diets containing PBP at different doses (two doses of PBP B1/day and three doses of PBP C1/day) compared with that containing monensin, but did not differ from the control diet.

The mean concentrations of NH_3-N in the rumen were influenced by the inclusion of PBP in the diet, but this was not observed in previous studies (Prado et al., 2010; Simioni, 2011). However, propolis appeared to reduce NH_3 production. Ozturk et al. (2010) investigated the effects of different concentrations of an ethanolic propolis extract on microbial fermentation *in vitro* and found that the concentration of NH_3-N in the rumen fluid was reduced to 24% and 39% of the control value with the addition of low and high concentrations of the propolis extract, respectively. Similarly, Oeztuerk et al. (2010) investigated the effects of nisin and propolis on *in vitro* fermentation of a 60:40 forage-to-concentrate diet and observed that both substances decreased NH_3 production. Likewise, Aguiar et al. (2013) observed that propolis extracts B1 and C1 displayed strong antimicrobial activity against hyper-ammonia-producing rumen bacteria; this may have contributed to the reduction of NH_3 production in the rumen.

It is important to emphasize the role of protozoa since they are known to be sensitive to PBP (Ríspoli et al., 2009). The most important aspect of protozoa is their ability to engulf large molecules, such as proteins, carbohydrates, and rumen bacteria (Van Soest, 1994). Because protozoa are not able to use NH_3-N, a fraction of the engulfed insoluble protein returns to the ruminal fluid in the form of soluble protein (Dijkstra, 1994). Thus, defaunation decreases the concentration of NH_3-N in the rumen (Eugene et al., 2004). Therefore, propolis-based products (especially PBP C1) act not only on NH_3-producing bacteria but also on protozoa.

Microbial protein synthesis and its efficiency were not affected by inclusion of propolis in the diet, similarly to what was observed in previous studies (Valero, 2010; Simioni, 2011; Aguiar et al., 2012). In dairy cows, allantoin and uric acid are secreted in the milk, and the amount secreted daily is equivalent to approximately 5% of the purine derivatives (PD) excreted in urine (Chen and Gomes, 1992). In our study, the corresponding value averaged 6.22%. The efficiency of microbial synthesis was not affected ($P>0.05$) by inclusion of PBP in the diet. According to the NRC (1996), 130.0 g/kg of TDN (microbial protein synthesis efficiency) is desirable, and only in the PBP C1 treatment was a comparable value attained.

The addition of the PBP did not affect any of the assayed blood parameters in the evaluated dairy cows. Similar results were reported by Faria et al. (2011), who found no effect of propolis (PBP C1 in two increasing doses) on blood urea concentrations in feedlot cattle. Similarly, Simioni (2011) reported no effect of propolis on blood parameters in feedlot cattle that were fed diets with higher propolis doses than those used by Faria 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). Dietary crude protein is the most

important nutritional factor that influences the MUN, and its determination can be used as a diagnostic of protein feeding in dairy cows (Nousiainen et al., 2004) and for the identification and/or correction of deficiencies, excesses, or imbalances in dietary protein and energy (Godden et al., 2001). In the present study, the mean milk protein concentration and MUN were 3.69% and 14.63 mg/dL, respectively. According to Godden et al. (2001), milk protein concentrations above 3.2% and MUN between 12 and 17 mg/dL indicate proper balance between the degradable protein and energy fermented in the rumen; the values obtained in our study are within these ranges.

For blood urea nitrogen (BUN), the obtained mean value was 18.72 mg/dL. Oliveira et al. (2001) found that BUN values between 19 and 20 mg/dL and MUN values between 24 and 25 mg/dL represent thresholds that indicate the initiation of N losses. The values obtained for BUN and MUN in our study are below these thresholds.

Conclusions

The propolis-based products have positive effects on protein metabolism in the rumen, without interfering with any other parameter evaluated. The propolis concentration and alcoholic level used in this study influence the amounts of flavonoids and phenolic acids in the propolis-based products, which may interfere with the observed effects on ruminal metabolism and digestive parameters.

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