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Effects of phenolic compounds on ruminal protozoa population, ruminal fermentation, and digestion in water buffaloes

E.M. de Paula [a], R.B. Samensari [b], E. Machado [b], L.M. Pereira [b], F.J. Maia [c], E.H. Yoshimura [b], R. Franzolin [d], A.P. Faciola [a,*], L.M. Zeoula [b]

[a] Department of Agriculture, Nutrition, and Veterinary Sciences, University of Nevada, Reno, NV, USA
[b] Department of Animal Sciences, Maringá State University, Maringá, PR, Brazil
[c] Department of Animal Sciences, Federal Technologic University of Paraná, Dois Vizinhos, PR, Brazil
[d] Department of Animal Sciences, University of Sao Paulo, Pirassununga, SP, Brazil

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ABSTRACT

The objective of this study was to evaluate different doses of phenolic compounds extracted from honey bees propolis on ruminal protozoa population, microbial protein synthesis, ruminal fermentation, solids' passage rate, and total tract digestibility in water buffaloes. Four crossbred non-lactating female water buffaloes averaging 543.9 ± 32.1 kg of body weight fitted with rumen cannulas were used in a 4 × 4 latin square design. The phenolic compounds were dosed in the rumen via rumen cannula in four doses: 0, 16.95, 33.9, and 50.85 mg/d; respectively. The total mixed ration consisted of 800 g/kg corn silage and 200 g/kg concentrate. Phenolic compounds linearly reduced the *Entodinium* protozoa population ($P < 0.01$) and quadratically increased ruminal acetate concentration ($P < 0.05$) and there was a trend to increase microbial protein synthesis in the rumen ($P = 0.07$). Phenolic compounds did not change total digestibility of dry matter and nutrients, solids' passage rate, and the efficiency of microbial protein synthesis ($P > 0.05$). It can be concluded that phenolic compounds reduce the *Entodinium* protozoa population in water buffaloes and change ruminal fermentation pattern, favoring acetate fermentation.

1. Introduction

Feed additives have the potential to improve nutrient utilization in farm animals by modifying ruminal microbial population and consequently ruminal fermentation and digestion (McGuffey et al., 2001). Public perception, safety concerns, and government regulations have imposed challenges to the use of synthetic feed additives in the diets of farm animals; therefore, alternative means of manipulating ruminal microbial population should receive especial attention. Propolis is a resinous mixture produced by honey bees and its extract is rich in phenolic compounds that possess antimicrobial activities that may improve nutrient utilization in the rumen. Previous studies have reported that propolis extract increased total tract digestibility of OM, NDF, and total carbohydrates (Ozturk et al., 2010; Prado et al., 2010; Costa Jr et al., 2012), decreased ruminal NH_3–N (Ozturk et al., 2010; Aguiar et al., 2014), and ruminal protozoa population (Morsy et al., 2015). However, phenolic compounds in propolis extract vary depending on honey bee species and available vegetation (Bankova, 2005). Researchers from Maringá State University developed a propolis extract (LLOS, PI 0605768-3) that showed promising results when dosed intra-ruminally to water buffaloes consuming a forage based diet (Costa Jr et al., 2012); however, this extract was obtained by lyophilization, which is a costly and labor intensive drying method. In the current study, a propolis extract obtained with spray drying methodology, which is less costly and more practical was tested in water buffaloes.

The objectives of this study were to evaluate the effects of different doses of phenolic compounds from propolis extract on ruminal protozoa population, microbial protein synthesis, ruminal fermentation, solids' passage rate, and total tract digestibility in water buffaloes. We hypothesized that phenolic compounds would suppress ruminal protozoa population and improve ruminal fermentation in water buffalos fed forage based diets.

2. Materials and methods

All experimental procedures were carried out at Maringá State University, Maringá, PR, Brazil and all animals were cared for according to the guidelines from the local animal care and use committee.

* Corresponding author.
E-mail address: afaciola@cabnr.unr.edu (A.P. Faciola).

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Table 1
Ingredient composition and chemical analysis of the basal diet.

Item	Total mixed ration
Ingredients, g/kg dry matter	
Corn silage	800
Corn meal	58
Wheat meal	80
Soybean meal	47
Urea	5
Vitamin mineral mix[a]	10
Chemical composition	
Dry matter, g/kg	421
Organic matter, g/kg DM	950
Crude protein, g/kg DM	89
Ether extract, g/kg DM	26
Neutral detergent fiber, g/kg DM	481
Acid detergent fiber, g/kg DM	291
Indigestible neutral detergent fiber, g/kg DM	11

[a] Provided (/kg DM): 56 mg of Zn, 46 mg of Mn, 22 mg of Fe, 12 mg of Cu, 0.9 mg of I, 0.4 mg of Co, 0.3 mg of Se, 6440 IU of vitamin A, 2000 IU of vitamin D, and 16 IU of vitamin E.

Table 2
Phenolic acids and flavonoids composition of propolis extract dried with spray-dryer.

Phenolic compounds (mg g^{-1})	Spray-dryer[a]
Phenolic acids	
Chlorogenic acid	n.d[c]
Caffeic acid	22.84
ρ-coumaric acid	39.13
CAPE[d]	11.82
Flavonoids	
Artepillin C	31.95
Apigenin	6.15
Pinocembin	3.94
Chrysin	3.49
Acacetin	4.81
Total phenolic compounds	124.13

[a] Quantification of flavonoids and phenolic acids using spray-drier method were performed using calibration curves obtained from apigenin and ρ-coumaric acid analytical standards, respectively (de Aguiar et al., 2013).
[c] Not detected.
[d] Caffeic acid phenethyl ester.

2.1. Animals, treatments, and experimental design

Four crossbreed non-lactating female water buffaloes (Murrah-Jafarabadi), averaging 543.9 ± 32.1 kg body weight (BW), fitted with rumen cannulas, were used in a 4 × 4 latin square design with four treatments and four 20-d periods, consisted of 14 days for diet adaptation and six days for sample collection. Animals were kept in individual stalls with individual feeders and *ad libitum* water supply. Total mixed ration (TMR) contained 800 g/kg corn silage and 200 g/kg concentrate (on a dry matter basis). The concentrate was based on corn meal, wheat meal, soybean meal, urea, and vitamin and mineral mix. The chemical composition of the diets is presented in Table 1.

The only difference among experimental treatments was the level of phenolic compounds inclusion. The control diet (diet A) had no added phenolic compounds and the other treatments had stepwise levels of phenolic compounds: 16.95 mg/d (diet B); 33.90 mg/d (diet C), and 50.85 mg/d (diet D). The phenolic compounds were dosed into the rumen via ruminal cannula twice a day, divided in two equal portions, at feeding times.

2.2. Propolis sample collection

The propolis samples were obtained from the apiary of the Iguatemi experimental station from Maringá State University, Paraná, Brazil. The apiary is located within a reserve of eucalyptus (*Eucalyptus* sp) surrounded by native forest predominantly consisted of *Baccharis dracunlifolia*. The raw propolis used for the extract preparation was collected from the apiary in a single day, from beehives of Africanized honey bees (*Apis mellifera*). Propolis samples were placed in plastic containers and stored at −22 °C until extracts preparation.

2.3. Propolis extracts preparation

The propolis extracts were prepared according to Franco and Bueno, (1999), briefly the extracts were obtained at propolis concentrations between [5–30% (w/v)] in water solutions [60.0–93.8 (v/v) of alcohol] by turbo extraction, for 15 min. The extracts were filtered under vacuum, the alcohol was removed in a rotary evaporator (Buchi, model RT 210, São Paulo, SP, Brazil) and then spray dried (nebulizer Labmaq, model MSD 1, Ribeirão Preto, SP, Brazil), with inlet temperatures of 100 °C. After drying, samples were stored in closed bottles and kept at −22 °C. Propolis extracts were registered in the Brazilian National Institute of Industrial Property, under number 0605768-3. The phenolic compounds were quantified using high performance liquid chromatography with photodiode array (HPLC-PDA) according to de Aguiar et al. (2013) and their chemical composition is presented in Table 2.

2.4. Data collection and sampling procedures

Animals were fed *ad libitum* twice daily at 0800 and 1600 h. Diets were adjusted daily to maintain orts at about 50–100 g/kg of offered TMR. Both diets and orts were collected and a representative composite sample was made per animal per treatment. Diets and orts samples were placed in plastic bags and stored at −10 °C for later chemical analysis.

From day 15 to day 19, fecal samples were collected, approximately 100 g were taken directly from the rectum at 0800 and 1600 h. Samples were dried in a ventilated oven at 55 °C for 72 h, ground at 1 mm mesh screen, and then mixed in equal proportions, based on the dry weight, to make the composite sample.

Solids' passage rate was determined as described by Udén et al., (1980), on day 15, 100 g of Cr-NDF labeled corn silage was dosed in the rumen and fecal samples (100 g) were taken at 0, 6, 12, 16, 24, 30, 36, 48, 72, 96, and 120 h after Cr-NDF labeled corn silage dosing.

On day 20, rumen fluid was collected via rumen cannula from four different locations in the rumen at 0, 2, 4, 6, and 8 h after morning feeding. Rumen pH was measured immediately after sampling and sub-samples of rumen fluid were acidified with 0.5 ml sulfuric acid 1:1 for NH_3–N and volatile fatty acids (VFA) analyses. All rumen fluid samples were labeled and kept in plastic bottles at −20 °C until analyses.

To determine ruminal microbial protein synthesis and efficiency, urine samples were collected on day 17 approximately four hours after feeding through mechanical stimulation of the vulva. To prevent purine derivate (PD) destruction due to uric acid precipitation, a 15 ml aliquot of urine was diluted with 5 ml 0.036 N sulfuric acid and samples were labeled and stored at 5 °C for subsequent analysis.

For ruminal protozoa population determination, extra samples of rumen content were obtained manually via rumen cannula approximately 30 min before feeding on day 18 and day 19. Samples were fixed and diluted with an equal volume of formalin solution (18.5% formaldehyde final concentration). Samples were stored at room temperature until later analysis.

2.5. Analytical procedures

Feeds, orts, and feces were analyzed according to AOAC, (1990) as follow: method 934.01 to determine dry matter (DM); method 924.05 to determine organic matter (OM); method 920.87 to determine crude protein (CP) and method 920.85 to determine ether extract (EE). Determination of neutral detergent fiber (NDF) was performed according to Van Soest et al. (1991), using a heat stable amylase, without sodium sulfite and expressed inclusive of residual ash, and for acid detergent fiber (ADF) expressed inclusive of residual ash according to method 973 from AOAC, (1990).

Total carbohydrates (TCHO) were calculated using the following equation: 100-(CP+EE+ash). To determine non-fibrous carbohydrates (NFC), NDF was subtracted from TCHO according to Sniffen et al. (1992). Indigestible NDF, which is the NDF remaining after a 12-d in situ incubation (Nocek, 1988), was used as an internal marker to estimate fecal output.

Chromium concentration in feces was determined by atomic absorption spectrometry according to Williams et al. (1962). A bi-compartmental model described by Grovum and Williams, (1973) was used to calculate solid's passage rate according to the equations proposed by Colucci et al. (1990).

Rumen fluid samples were filtered to determine ruminal NH_3–N concentration (Vieira, 1980). Individual VFA concentrations were determined according to Palmquist and Conrad (1971) using liquid–gas chromatography (Finnigan 9001), packed with glass column (4% CW 20 M-80/120 Carbopack B-DA) of 2 m × 1/8" associated with an integrator.

To determine purine derivatives (PD) concentration, urine allantoin analysis were performed according to Chen and Gomes, (1992). Creatinine and uric acid were determined using commercials kits (Analisa®) and readings were conducted using a spectrophotometer (Shimadzu model UV-1601).

Urinary volume (L) was estimated from creatinine concentration (mg/L), daily excretion of creatinine (mg/kg BW) was divided by creatinine concentration (mg/L). To determine daily creatinine excretion (mmol/kg $BW^{0.75}$), 0.44 mmol/kg $BW^{0.75}$ was used according to Chen et al. (1996). Microbial nitrogen was calculated from amounts of purine absorption (X mmol/d) which was estimated from PD urinary excretion (Y mmol/d) calculated according to the equation described by Dipu et al. (2006) for water buffaloes $Y=0.74x+(0.117\ BW^{0.75})$. The 0.74 value represents purine that was recovered from PD urine absorption and 0.117 mmol/kg $BW^{0.75}$/d represents PD from endogenous contribution. Rumen microbial nitrogen synthesis (Y g of N/d) was calculated from the purine absorption ratio (X mmol/d) using the equation: $Y=((X\ (\text{mmol/d})\times 70)/(0.116\times 0.83\times 1000))$ (Chen and Gomes, 1992). Considering that 70 represents purine nitrogen content (mg of N/mmol); 0.83 represents microbial purine digestibility and 0.116 represents total rumen microorganism N-purine:N ratio.

Microbial protein synthesis (MPS) was obtained by multiplying microbial N by 6.25, while the efficiency of microbial protein synthesis (EMPS) was determined as: EMPS=MPS(g)/100 g of total digestible nutrients (TDN).

Total counts of ruminal protozoa and generic distribution were done in 100 microscopic fields at magnification of 100 × according to Dehority, (1993).

2.6. Statistical analysis

Data were analyzed in a 4 × 4 latin square design with diet, period, and cow as factors using the MIXED procedures of (SAS, 2002). The statistical model used was:

$$Y_{ijk} = \mu + A_i + P_j + T_k + e_{ijk},$$

Where Y_{ijk}=observed effect of treatment k, on period j, in animal i; A_i=effect of animal; P_j=effect of period; T_k=effect of the treatment; e_{ijk}=random error associated with each observation. Orthogonal polynomial contrasts were used to examine the responses (linear and quadratic) to increasing doses of phenolic compounds in the treatments.

Analyses of VFA, pH, and NH_3-N were done as repeated measures in the MIXED procedures of SAS (2002), considering the effects of animal, period, treatment, time, and treatment*time interaction in the model. Significant differences were declared at $P \leq 0.05$. Trends were discussed at $P < 0.10$.

3. Results

3.1. Phenolic compounds composition

Phenolic compounds present in propolis extract were identified and quantified in this study; they are listed in Table 2 and include caffeic acid, ρ-coumaric acid, and caffeic acid phenethyl ester (CAPE). the most abundant phenolic acid quantified was ρ-coumaric acid at 39.13 mg g^{-1} of dried extract. the flavonoids identified and quantified were artepillin C, apigenin, pinocembrim, chrysin and acacetin, the most abundant flavonoid quantified was artepelin C at 31.95 mg g^{-1}.

3.2. Intake, apparent digestibility, and solids' passage rate

Dry matter intake (DMI) and apparent digestibility of nutrients were not affected by increased doses of phenolic compounds ($P > 0.05$) and results are presented in Table 3. there was a negative linear trend ($P=0.10$) for CP apparent digestibility and the greatest value was observed in diet a. also, there were no differences among treatments for solids' passage rate ($P > 0.05$), Table 4.

3.3. Rumen fermentation

There were no differences in ruminal propionate and butyrate concentrations as well as in acetate: propionate ratio ($P > 0.05$); nevertheless, a quadratic response was observed in acetate concentration ($P=0.04$), treatments B and C increased ruminal acetate molar proportion by about 4% compared with the control treatment and 10% compared with treatment D (Table 5). It was also

Table 3
Effects of phenolic compounds on dry matter intake and apparent digestibility in water buffaloes.

Item	Treatment				SEM	*P*-value		
	A[a]	B[b]	C[c]	D[d]		TRT[e]	L[f]	Q[g]
Dry matter intake								
kg/d	7.8	7.9	7.9	8.1	0.14	0.50	0.17	0.71
g/kg BW	14.2	14.3	14.4	14.8	0.04	0.46	0.18	0.75
g/kg $BW^{0.75}$	68.6	69.1	69.5	71.0	1.87	0.36	0.14	0.50
Apparent digestibility g/kg								
Dry matter	707	712	709	698	8.96	0.42	0.28	0.22
Organic matter	718	723	720	709	8.10	0.47	0.31	0.25
Crude protein	691	655	660	658	10.8	0.14	0.10	0.15
Neutral detergent fiber	588	598	599	580	11.2	0.45	0.59	0.16
Non fiber carbohydrates	896	899	895	892	6.41	0.70	0.38	0.51

[a] A: treatment without addition of phenolic compounds.
[b] B: treatment with 16.95 mg of total phenolic compounds/day.
[c] C: treatment with 33.90 mg of total phenolic compounds/day.
[d] D: treatment with 50.85 mg of total phenolic compounds/day.
[e] Overall treatment effect.
[f] Linear effect of different doses of phenolic compounds.
[g] Quadratic effect of different doses of phenolic compounds.

Table 4
Effects of phenolic compounds on solids' passage rate in water buffaloes.

Item	Treatment				SEM	*P*-value		
	A[a]	B[b]	C[c]	D[d]		TRT[e]	L[f]	Q[g]
k1[h], h^{-1}	0.03	0.04	0.04	0.04	0.39	0.61	0.25	0.75
k2[i], h^{-1}	0.18	0.18	0.17	0.17	0.79	0.69	0.28	0.94
RRRT[j], h	31.2	28.5	29.1	28.0	3.55	0.58	0.32	0.63
PRRT[k], h	5.7	5.6	6.0	6.0	0.25	0.63	0.26	0.81
MRT[l], h	36.9	34.1	35.0	34.1	3.64	0.65	0.43	0.61
TT[m], h	18.5	16.9	19.5	19.9	2.39	0.81	0.50	0.69
TRT[n], h	55.4	50.9	54.6	53.9	3.60	0.79	0.93	0.58

[a] A: treatment without addition of phenolic compounds.
[b] B: treatment with 16.95 mg of total phenolic compounds/day.
[c] C: treatment with 33.90 mg of total phenolic compounds/day.
[d] D: treatment with 50.85 mg of total phenolic compounds/day.
[e] Overall treatment effect.
[f] Linear effect of different doses of phenolic compounds.
[g] Quadratic effect of different doses of phenolic compounds.
[h] Ruminal-reticulum passage rate.
[i] Post-ruminal passage.
[j] Ruminal-reticulum retention time.
[k] Post-ruminal retention time.
[l] Mean retention time.
[m] Transit time.
[n] Total retention time.

Table 5
Effects of phenolic compounds on ruminal fermentation in water buffaloes.

Item	Treatments				SEM	*P*-value		
	A[a]	B[b]	C[c]	D[d]		TRT[e]	L[f]	Q[g]
Total VFA, mmol	74.6	76.0	76.7	69.6	3.81	0.14	0.19	0.07
Acetate, mmol/100 mmol	51.5	53.4	53.3	48.1	2.59	0.10	0.18	0.04
Propionate, mmol/100 mmol	13.7	14.1	14.3	12.7	0.71	0.27	0.31	0.12
Butyrate, mmol/100 mmol	9.30	8.51	9.10	8.81	0.74	0.40	0.71	0.46
Acetate:Propionate	3.86	3.82	3.79	3.86	0.08	0.90	0.91	0.51
NH_3-N, mg/100 mL	9.58	8.81	9.63	9.70	1.18	0.56	0.51	0.41
pH	6.78	6.76	6.88	6.96	0.06	0.08	0.02	0.36

[a] A: treatment without addition of phenolic compounds.
[b] B: treatment with 16.95 mg of total phenolic compounds/day.
[c] C: treatment with 33.90 mg of total phenolic compounds/day.
[d] D: treatment with 50.85 mg of total phenolic compounds/day.
[e] Overall treatment effect.
[f] Linear effect of different doses of phenolic compounds.
[g] Quadratic effect of different doses of phenolic compounds.

observed a trend for a quadratic response in total ruminal VFA concentration ($P=0.07$) in a similar pattern observed for ruminal acetate. Ruminal NH_3–N concentration was not affected by treatments. There was a positive linear response of ruminal pH to increasing levels of phenolic compounds ($P<0.05$).

3.4. Ruminal protozoa population

The mean concentration and composition of ruminal protozoa population are presented in Table 6. Ruminal protozoa from the genus *Entodinium*, *Epidinium*, and *Isotricha*, and also from the sub-family Diplodiniinae were observed; however, ruminal protozoa from the genus *Dasytricha* and *Choronina* were not observed in this experiment.

The *Entodinium* population was linearly reduced by increased doses of phenolic compounds ($P=0.01$). Compared with the control treatment, treatments B, C, and D reduced protozoa population by

Table 6
Effects of phenolic compounds on ruminal protozoa population in water buffaloes.

Item	Treatment				SEM	*P*-value		
	A[a]	B[b]	C[c]	D[d]		TRT[e]	L[f]	Q[g]
	Cell $\times 10^5$/mL							
Entodinium	1.59	1.41	1.36	1.16	0.14	0.07	0.01	0.93
Diplodiniinae	4.13	2.83	2.81	3.28	0.34	0.34	0.34	0.14
Epidinium	0.08	0.16	0.12	0.08	0.03	0.21	0.55	0.07
Isotricha	0.04	0.05	0.02	0.05	0.02	0.72	0.84	0.84
Total	5.85	4.47	4.32	4.57	0.70	0.29	0.18	0.20
Ento:Diplo	0.45	0.53	0.49	0.39	0.07	0.56	0.49	0.24
	% of total							
Entodinium	29.6	32.5	32.1	26.9	3.52	0.61	0.58	0.25
Diplodiniinae	68.7	63.6	65.0	71.0	3.84	0.43	0.59	0.14
Epidinium	1.55	3.80	2.85	2.00	0.81	0.21	0.98	0.07
Isotricha	0.57	1.12	0.51	1.10	0.36	0.52	0.71	0.96

[a] A: treatment without addition of phenolic compounds.
[b] B: treatment with 16.95 mg of total phenolic compounds/day.
[c] C: treatment with 33.90 mg of total phenolic compounds/day.
[d] D: treatment with 50.85 mg of total phenolic compounds/day.
[e] Overall treatment effect.
[f] Linear effect of different doses of phenolic compounds.
[g] Quadratic effect of different doses of phenolic compounds.

11, 14, and 27%, respectively. There were no differences in protozoa population from the *Isotricha* genus and from the sub-family Diplodiniinae ($P>0.05$). However it was observed a trend for a quadratic response in the *Epidinium* genus ($P=0.07$). Diplodiniinae was the most abundant genus among all treatments.

3.5. Microbial protein synthesis

There were no differences in microbial protein synthesis and microbial protein synthesis efficiency ($P>0.05$), Table 7. Nonetheless, microbial protein synthesis had a linear positive trend ($P=0.07$) when phenolic compound were dosed in the rumen, microbial protein synthesis in treatment D was 44% higher when compared to the control treatment.

Table 7
Effects of phenolic compounds on ruminal microbial synthesis in water buffaloes.

	Treatments				SEM	*P*-value		
	A[a]	B[b]	C[c]	D[d]		TRT[e]	L[f]	Q[g]
Urine volume, L	19.9	21.2	21.5	20.7	3.87	0.94	0.77	0.61
Creatinine, mmol/L	14.2	12.7	12.7	14.5	1.59	0.47	0.20	0.47
Purine derivatives, mmol/d								
Allantoin excretion	74.6	81.7	71.4	102.5	8.25	0.09	0.07	0.16
Uric acid excretion	1.90	1.97	1.98	2.10	0.23	0.35	0.11	0.69
PD[h] absorbed	85.2	94.7	80.1	123.1	10.9	0.08	0.06	0.15
Microbial protein, g/d								
MPS[i]	386.9	431.1	368.0	559.3	49.3	0.09	0.07	0.16
EMPS[j]	7.16	9.03	7.06	10.2	1.22	0.24	0.20	0.60

[a] A: treatment without addition of phenolic compounds.
[b] B: treatment with 16.95 mg of total phenolic compounds/day.
[c] C: treatment with 33.90 mg of total phenolic compounds/day.
[d] D: treatment with 50.85 mg of total phenolic compounds/day.
[e] Overall treatment effect.
[f] Linear effect of different doses of phenolic compounds.
[g] Quadratic effect of different doses of phenolic compounds.
[h] Purine derivatives.
[i] Microbial protein synthesis.
[j] Efficiency of microbial protein synthesis (g MPS^F/100 g of TDN).

4. Discussion

4.1. Phenolic compounds composition

Phenolic compounds in propolis extract have been studied as a potential alternative to synthetic additives in ruminant's diets. However, propolis chemical composition may vary due to several reasons, including the different plant community characteristics in which the bees live (Popova et al., 2010), and the extraction method with different solvents or different solvent concentration, which may yield different compounds that affect the biological activity of the propolis extract (Sforcin and Bankova, 2011). Therefore these studies should take into account the phenolic compounds chemical composition in order to better understand its effect on nutrition and metabolism (de Aguiar et al., 2013).

In the present study the most abundant phenolic acid and flavonoid were ρ-coumaric and artepelin C; respectively. Costa Jr. et al. (2012) also identified and quantified the phenolic compounds in propolis extract, the propolis extract identified as LLOS C1 was extracted with the same solvent, the same solvent concentration, and from the same geographical location of the propolis extract evaluated in the present study. They also found the phenolic acid ρ-coumaric acid and the flavonoid artepelin C as the most abundant among the phenolic compounds; however, some phenolic compounds were found in different concentration compared to our study. In addition, they found the phenolic acid chlorogenic acid and the flavonoid galangin, whereas these phenolic compounds were not detected in our samples. Morsy et al. (2015) compared the in vitro efficiency of supplementary propolis extract of different origin (Brazilian or Egyptian) on ruminal degradability of OM and methane formation. The most abundant compounds found in both propolis were isoflavoinoids. Nevertheless, in the Brazilian propolis they found chalcones compounds, whereas in the Egyptian they did not found chalcones compounds, but they found saturated fatty acids. Compared to our findings the propolis extract from their experiment were widely different in their chemical composition, which may cause different effect on digestion and metabolism.

4.2. Intake, apparent digestibility, and solids passage rate

In the current study it was not observed significant differences in DMI when phenolic compounds were dosed in the rumen, which agrees with previous studies that used similar extracts in the diets of water buffaloes (Prado et al., 2010; Costa Jr. et al., 2012). This suggests that phenolic compounds from propolis may not affect DMI when dosed into the rumen at levels up to 50 mg/d. In the present study it was not observed differences among treatments for apparent digestibility of nutrients, which is contrary to previous studies that observed an increase in nutrient digestibility (Prado et al., 2010; Costa Jr. et al., 2012). This discrepancy may have been due to different chemical composition of the extracts used in previous studies, which were obtained via lyophilization as opposed to spray drying, which altered the composition and concentration of the phenolic compounds. Lyophilization reduced the concentration of phenolic acids and increased the concentration of flavonoids compared to spray drying. According to Bankova (2005), differences in propolis extract phenolic acids composition may interfere with its biological activity. Solids' passage rate was not affected by the treatments and this may be a consequence of the lack of changes in DMI and nutrient digestibilities.

4.3. Ruminal fermentation

Phenolic compounds significantly changed ruminal fermentation, a quadratic response was observed in acetate molar proportion and total VFA concentration. These findings are in agreement with Costa Jr. et al. (2012) and Morsy et al. (2015) that also observed greater total VFA concentration and greater molar proportion of acetate when propolis extract was added in the diets. Because nutrient digestibilities did not change in this study, it is likely that changes in microbial populations may have contributed to the differences observed in ruminal acetate and total VFA concentration. An increase in total VFA concentration is very desirable from an energetic standpoint because VFA are an important energy substrate in ruminants. Acetate plays a major role in fat synthesis; therefore, acetate formation is desirable in water buffaloes which are notorious for their high milk fat. Moreover, acetate formation may be an indication of healthy ruminal fermentation which is important for the animal's production and wellbeing.

Despite the observed changes in ruminal pH it is unlikely that the magnitude of the differences observed have practical implications.

4.4. Ruminal protozoa population

Literature data reporting the effects of phenolic compounds on ruminal protozoa population are scarce. Akin (1982) have found that ρ-coumaric acid had a toxic effect on rumen protozoa. In the present study there was a significant linear reduction of the *Entodinium* protozoa population (Table 7). In agreement with our findings, Ríspoli et al. (2009) found a significant reduction of *Entodinium* in the rumen of water buffaloes fed propolis extract compared to a control diet.

Entodiniomorphids protozoa, such as, *Entodinium* and *Epidinium* are capable of ingesting and digesting insoluble proteins, as well as bacterial proteins (Jouany, 1996); therefore, suppressing these protozoa may reduce NH_3–N formation in the rumen, which was not observed in the present study. Dietary CP plays an important role on ruminal NH_3–N concentration and typically a reduction in ruminal protozoa population improves N metabolism when diets are limiting in N (Jouany, 1996) and this may not have been the case in the present study. Phenolic compounds may improve N metabolism when diets are limiting in N due to its effects on ruminal protozoal population.

The Diplodiniinae genus was the most abundant genus observed in all treatments and this result is in agreement with Franzolin and Dehority, (1999) that also found greater Diplodiniinae population in water buffaloes fed forage based diet. The Diplodiniinae population had a 30% reduction in treatment B and C, and a 20% reduction compared to the control treatment; however, these were not statistically significant ($P > 0.05$).

4.5. Microbial protein synthesis

The linear trend to increase microbial protein synthesis may be related to the significant ($P < 0.05$) linear reduction in the *Entodinium* population numbers. Studies have shown that decreasing protozoa population may cause an increase in the flow of bacterial nitrogen in the duodenum (Jouany et al., 1988). Koening et al. (2000) concluded that defaunation improves the intraruminal metabolism of N by increasing ruminal bacterial biomass synthesis and flow of bacteria to the intestine.

Microbial protein synthesis and efficiency were not affected in previous studies when propolis extract were added to ruminants' diets (Costa Jr et al., 2012; Aguiar et al., 2014); however, in the present study it was observed an increase of 12% for treatment B and 44% for treatment D for MPS, compared with the control treatment. This discrepancy may have been caused by differences in phenolic compounds associated with different drying methods.

5. Conclusion

Phenolic compounds intra-ruminally dosed at 16.9 and 33.9 mg/d increased ruminal acetate molar proportion. Phenolic compounds also reduced ruminal *Entodinium* population in water buffaloes. Moreover, phenolic compounds tended to increase microbial protein synthesis and total VFA concentration in the rumen of water buffaloes. These results indicate that phenolic compounds present in propolis extract may improve ruminal fermentation especially when high forage and low N diets are fed due to its effect on ruminal fermentation and protozoa population. Moreover, higher acetate concentration may be desired in water buffaloes due to acetate's role in fat synthesis. The results from this study also show that propolis extract may have different phenolic compounds which will affect its biological properties and its effects on nutrition and metabolism.

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