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Antioxidant action in diets with ground soybeans on ruminal microbial production, digestion, and fermentation in buffaloes

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ABSTRACT - The objective of this study was to evaluate the effect of adding ground soybeans and antioxidants to diet of buffaloes on intake, ruminal microbial production, total and ruminal digestibility of nutrients, and ruminal fermentation parameters. Four crossbred buffaloes with a mean weight of 506±29 kg were distributed in a 4×4 Latin square. Four diets were tested: control; diet with ground soybeans; diet with ground soybeans and supplementation with yerba mate; and diet with ground soybeans and supplementation with yerba mate and vitamin E. The addition of ground soybeans had negative effects on intake of dry matter, organic matter, crude protein, neutral detergent fiber, and acid detergent fiber, as well as ruminal digestibility of neutral detergent fiber and total carbohydrates but did not influence total digestibility of nutrients. Yet, the ground soybeans diet increased the concentration of butyrate and microbial production in the rumen. The diet with ground soybeans supplemented with yerba mate decreased the concentration of acetate and increased the concentration of propionate and increased the efficiency of synthesis and the production of microbial protein in the rumen. There was a positive additive effect of vitamin E in the presence of yerba mate, enhancing the synthesis and production efficiency of microbial protein. Thus, the addition of ground soybeans and antioxidants to diet of buffaloes improves the efficiency of microbial protein synthesis and increases the production of butyrate, without, in general, altering the total digestibility of nutrients.

Keywords: ether extract, polyphenols, ruminal manipulation, ruminal metabolism, tocopherol

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Introduction

The objectives of supplying lipids in the diet of ruminants are to increase the energy density of diets, increase production, and improve lipid profile of milk, as well as to reduce incidence of ketosis, improve feed conversion, reduce caloric stress, and re-establish estrous cycle. However, lipid use may have detrimental effects on ruminal fermentation, especially on fibrolytic bacteria, and may cause a reduction in fiber degradability. Moreover, lipid sources with high levels of unsaturated fatty acids are prone to oxidation (Liener, 1994).

Antioxidants are important because they act to prevent oxidative reactions both in the feed and in animal as well in final products (Petit, 2015). Among antioxidants evaluated in ruminant nutrition, vitamin E is the most studied (Shakirullah et al., 2017), and results are positive. Vitamin E acts at the cell membrane level by inhibiting natural peroxidation of polyunsaturated fatty acids, while maintaining cell integrity.

Ilex paraguariensis A. St. Hill, commonly known as yerba mate (YM) is a tree belonging to the family *Aquifoleaceae*. It is native to South America and is of great cultural and economic importance for southern Brazil (Carelli et al., 2011), where it is one of the main agricultural products. Although YM is used currently only for the preparation of beverages such as mate, tea, and soft drinks, it has great potential for alternative uses due to the diversity of its phytochemical composition. One of most important classes of bioactive components present in YM is polyphenols, which are mainly natural hydrophilic antioxidants, such as flavonoids, tannins, and derivatives of caffeic acid (Filip et al., 2009). Beneficial properties of phenolic compounds can be attributed to their ability to neutralize free radicals, thus inhibiting the lipid peroxidation process.

Plant secondary metabolites such as polyphenols, essential oils, and saponins have been extensively evaluated for their role in microbial fermentation in the rumen. They have been shown to affect fermentation in the rumen to varying degrees depending on the source and concentration in diets (Benchaar et al., 2008). Plant phytogenic substances have been evaluated in ruminant nutrition and have shown to result in a reduction in methane emissions (Klevenhusen et al., 2011), better animal performance and health, and increased efficiency of nitrogen utilization (Celi, 2010). Thus, the hypothesis is that the combination of antioxidants (yerba mate and vitamin E) may improve the negative effects caused in ruminal metabolism by the addition of lipid source to buffalo diet. This study aimed to evaluate the effect of lipid and antioxidant supplementation in diets on ruminal and total nutrient digestibility, microbial production, and ruminal parameters in buffaloes.

Material and Methods

The research was carried out in Maringá, PR, Brazil (Latitude: 23°25'38" S; Longitude: 51°56'15" W). Experimental protocols developed in this study fully complied with Ethical Principles of Animal Experimentation, elaborated by the Brazilian College of Animal Experimentation (COBEA), and were approved by the local Ethics Committee on Animal Use (case no. 009/2013). The experiment was performed at an experimental farm in Iguatemi (PR, Brazil).

Four cannulated crossbred female buffaloes (*Bubalus bubalis*), with a mean weight of 506 kg±29 kg were assigned to a 4×4 Latin square, composed of four animals, four treatments, and four treatment periods. Animals were housed in individual stalls (9 m^2/animal), each containing water drinker, feed trough, and concrete floor. The experiment was conducted in four periods of 19 days each, with 14 days for adaptation of animals to the experimental diets and five days for data collection, comprising 76 days.

The diet consisted of a control ration, without addition of ground soybeans and a test ration formulated with the inclusion of ground soybeans (Table 1). At the time of feeding, to the corresponding treatments, vitamin E and/or dry YM leaves (Laranjeiras, Laranjeiras do Sul, PR, Brazil) were added directly into the rumen. Experimental diets (Table 1) were formulated to meet nutritional requirements of buffaloes in maintenance (Paul and Lal, 2010) and intake was *ad libitum*, allowing 50-100 g/kg orts, as fed. Four experimental diets were included: control - diet without lipid supplementation and without antioxidants; GS - diet with ground soybean grains (equivalent to 60 g oil/kg DM); GSYM - diet with ground soybean grains supplemented with YM (30 g/kg DM); and GSYME - diet with ground soybean grains supplemented with YM (30 g/kg DM) and vitamin E (375 IU/kg DM).

Animals were fed twice daily (8:00 and 16:00 h) and were weighed at the beginning of adaptation period and at the end of the collection period to calculate the amount of feed to be given.

The fatty acid composition of the oil extracted from the soybean grain and the concentrations of total polyphenols and flavonoids present in the mate grass are presented in table 2. The extraction of the fatty acids from the soybean was done with chloroform, methanol, and water in the proportion 2:2:1.8, as described by Bligh and Dyer (1959). The fatty acid methyl esters were separated by a gas chromatograph (Thermo, Trace Ultra 3300 model), equipped with a fused silica capillary column (CP-7420, Select FAME, 100 m × 0.25 mm d.i and 0.25 μm cyanopropyl) and flame ionization detector Martins et al. (2008).

The analysis of total polyphenols was performed by the colorimetric method described by Singleton and Rossi (1965), and the results obtained were expressed in mg EAG (equivalent to gallic acid).

The quantification of the flavonoids was performed by the colorimetric method described by Woisky and Salatino (1998) with modifications, and the results obtained are expressed in mg of EQ (equivalent to quercetin).

Feed samples were collected on days 15 to 19 and stored for further analysis of chemical composition. Fecal samples were collected at 8:00 and 16:00 h, directly from the rectum, on days 15 to 19. Omasal

Table 1 - Ingredient and chemical composition of experimental diets

	Diet[1]			
	Control	GS	GSYM	GSYME
Ingredient (g/kg DM)				
Pelletized sugarcane bagasse	250.00	250.00	250.00	250.00
Corn silage	550.00	550.00	550.00	550.00
Soybean meal	129.00	-	-	-
Ground soybean	-	174.50	174.50	174.50
Corn	61.00	15.50	15.50	15.50
Limestone	4.00	4.00	4.00	4.00
Mineral suplemment[2]	6.00	6.00	6.00	6.00
Yerba mate	-	-	30.00	30.00
Vitamin E (IU/kg DM)	-	-	-	375
Total	1000	1000	1000	1000
Chemical analysis				
Dry matter (g/kg fresh weight)	570.18	570.69	570.69	570.69
Organic matter (g/kg DM)	936.36	937.20	937.20	937.20
Crude protein (g/kg DM)	112.80	112.30	112.30	112.30
Ether extract (g/kg DM)	23.10	53.30	53.30	53.30
Neutral detergent fiber (g/kg DM)	520.31	518.16	518.16	518.16
Acid detergent fiber (g/kg DM)	329.05	329.33	329.33	329.33
Total digestible nutrients (g/kg DM)[3]	628.61	631.96	631.92	631.90

DM - dry matter.
[1] GS - diet with ground soybean grains; GSYM - diet with ground soybean grains supplemented with yerba mate; GSYME - diet with ground soybean grains supplemented with yerba mate and vitamin E.
[2] Composition of mineral supplement (per kg of product): 181 g of calcium; 130 g of phosphorus; 9.40 g of sulfur; 100 mg of cobalt; 1.25 g of copper; 2.20 g of iron; 90 mg of iodine; 2 g of manganese; 15 mg of selenium; 5.27 g of zinc; 1.3 g of fluorine.
[3] Tabulated values.

Table 2 - Fatty acid composition (g/kg dry matter) of oil extracted from soybeans and concentrations of total polyphenols and flavonoids in yerba mate

Oil extracted from soybean	
16:0	118.0
18:00	20.0
Cis9 18:1	258.0
Cis6 18:2	545.0
Cis3 18:3	46.0
Unidentified	13.0
Phenolic compounds of yerba mate	
Total polyphenols (gallic acid equivalent mg/g)	22.2
Flavonoids (quercetin equivalent mg/g)	2.5

digesta samples were collected by suction, according to the technique described by Huhtanen et al. (1997) and were performed every 27 h from days 15 to 19. Samples were stored in properly identified plastic bags and frozen.

Titanium dioxide (TiO_2) was used as an external marker for daily flow of dry matter of omasum and feces. Two intra-ruminal daily doses (8:00 and 16:00 h) of 7.5 g/dose of TiO_2, previously weighed and wrapped in hygroscopic paper, were provided for each animal (Myers et al., 2004).

Ruminal fluid was collected manually via the ruminal cannula before the first feeding (8:00 h), which was set as time zero (0), and at 2, 4, 6, and 8 h post-feeding to determine the pH and concentrations of ammonia nitrogen ($N\text{-}NH_3$) and short-chain fatty acids (SCFA). The pH was measured immediately after collection, then an aliquot of ruminal liquid was acidified with sulfuric acid (1:1) to determine concentration of ammonia nitrogen ($N\text{-}NH_3$), and another aliquot was frozen for determination of SCFA.

Cobalt-EDTA was used as a liquid marker to estimate ruminal dilution rate (Uden et al., 1980). Co-EDTA in solution (30 g of Co-EDTA diluted in 500 mL of distilled water) was administered in the rumen before the first feeding. Ruminal fluid was collected via ruminal cannula at time zero (before the first feeding) and at 2, 4, 6, 8, 10, 12, 14, 16, and 24 h post-feeding in the morning and stored at −15 °C to determine cobalt concentration. Urine spot samples were collected to determine purine derivatives and estimate efficiency of microbial protein. Urine was collected 4 h post-feeding, and an aliquot (10 mL) was stored in properly identified flasks containing 40 mL H_2SO_4 (0.036N).

Samples of feed, feces, and orts were dried at 55 °C for 72 h, individually ground to 1 mm, and mixed in equal amounts, based on dry weight, to form pooled samples of feces per animal and per treatment. Samples were chemically analyzed. Dry matter was evaluated in a forced-air oven according to the procedure 934.01 of AOAC (1990); ash was determined by combustion at 600 °C for 6 h according to method 924.05 of AOAC (1990); measurement of total nitrogen followed procedure 990.03 of AOAC (1990) and multiplied by 6.25 for determination of crude protein (CP); concentrations of neutral detergent fiber (NDF) were measured using thermostable amylase, without sodium sulphite (Mertens, 2002); concentrations of acid detergent fiber (ADF), without correction of the ash content, were determined according to procedure 973.18 of AOAC (1990); ether extract (EE) was determined according to the method 7.060 of AOAC (1990); non-fibrous carbohydrates (NFC) were determined according to equation: NFC (g/kg) = 1000 – CP (g/kg) + NDF (g/kg) + EE (g/kg); total carbohydrates were estimated according to the equation: 1000 – (CP (g/kg) + EE (g/kg) + Ash (g/kg)); and total digestible nutrients (TDN) of the diets were estimated according to the equation: TDN (g/kg) = digestible NFC (g/kg) + digestible CP (g/kg) + digestible NDF (g/kg) + (digestible EE, g/kg, × 2.25).

The digestibilities were calculated in relation to the amount of nutrient that arrived at the compartment. In this way, the equations used were:

Rumen digestibility:

$$\frac{\text{Nutrient intake} - \text{Nutrient concentration in omasal content}}{\text{Nutrient intake}}$$

Intestinal digestibility:

$$\frac{\text{Concentration of nutrients in omasal content} - \text{Concentration of nutrients in feces}}{\text{Concentration of nutrients in omasal content}}$$

Total digestibility:

$$\frac{\text{Nutrient intake} - \text{Nutrient concentration in feces}}{\text{Nutrient intake}}$$

Ammonia nitrogen ($N\text{-}NH_3$) in ruminal liquid was measured by distillation with potassium hydroxide (2N) (Preston, 1995). Short-chain fatty acids analysis was performed according to the technique

described by Palmquist and Conrad (1971), using a gas chromatograph (Shimadzu GC-2014, Kyoto, Japan) with a packed glass column (Carbopack B DA 4% Carbowax 20M, Supelco, São Paulo, SP, Brazil) of 2.0 m × 1/8", coupled to an integrator and a microcomputer.

Dilution rate, recycling time, and flow rate were estimated using parameter values from a linear regression equation using the natural log of cobalt concentrations (mg/100 mL) in ruminal fluid samples collected at different times. Dilution rate was represented by the regression coefficients obtained from the equations for each treatment and time evaluated. From these parameters (dilution rate and ruminal volume), recycling time (100/dilution rate) and ruminal flow (ruminal volume × dilution rate/100) were estimated.

Purine derivatives in urine were determined by allantoin analysis according to methodology described by Chen and Gomes (1992), and creatinine and uric acid were determined using commercial kits (Analisa, Belo Horizonte, MG, Brazil) and spectrophotometer (Shimadzu UV-1601, São Paulo, SP, Brazil).

Urine volume (L/day) was estimated from the ratio between the creatinine daily excretion (mmol/kg $LW^{0.75}$) and the creatinine concentration (mmol/L). The creatinine daily excretion was determined by considering the mean value of 0.44 mmol/kg $LW^{0.75}$ obtained by Chen et al. (1996) in buffaloes. The microbial nitrogen production was calculated from the amount of absorbed purines (X, mmol/day), which was in turn estimated from urinary excretion of purine derivatives (PD) (Y, mmol/day), using the equation described by Dipu et al. (2006) for buffaloes: $Y = 0.74X + (0.117\ LW^{0.75})$.

The synthesis of microbial nitrogen compounds in the rumen (Y, g N/day) was estimated as a function of absorbed purines (X, mmol/day), using the equation described by Chen and Gomes (1992): Y = X (mmol/day) × 70 / 0.116 × 0.83 × 1000.

The microbial protein synthesis (MPS) was determined by multiplying microbial N synthesis by 6.25, while the efficiency of microbial protein synthesis (EMPS) was determined using: EMPS (g/100 g) = MPS (g)/DOMR (100 g), in which DOMR = digestible organic matter fermented in the rumen.

The experimental design was a 4×4 Latin square, with four treatments and four experimental periods.

The mathematical model used was:

$$Yijk = \mu + Ai + Pj + Tk + eijk,$$

in which Yijk = observed variables; μ = overall mean; Ai = effect of animal i; Pj = effect of period j; Tk = treatment effect k; and eijk = random error.

Results were analyzed using the MIXED procedure of statistical software SAS (Statistical Analysis System, version 9.0). Significance was declared at $P<0.05$, and trends were accepted at $P\leq0.10$. Orthogonal contrasts were used to compare the effects of: (1) soybean grain (control versus GS, GSYM, and GSYME); (2) yerba mate (GS versus GSYM and GSYME); and (3) the association between the yerba mate and vitamin E (GSYM versus GSYME).

Analyses of VFA, pH, and NH3-N were carried out as repeated measures in the MIXED procedures of SAS, considering the effects of animal, period, treatment, time, and treatment × time interaction in the model. Significant differences were declared at $P\leq0.05$.

Results

Inclusion of ground soybeans in the diet affected intake ($P<0.05$) of all evaluated nutrients, except total carbohydrates (TC) (Table 3), with reductions in DM, organic matter (OM), CP, NDF, and ADF, and increased intake of EE. Combining antioxidants (YM and vitamin E) reduced the intake of NDF. In addition, a negative effect of soybeans was observed on ruminal digestibility of NDF and TC. For the other treatments, there was no effect on ruminal, intestinal, and total digestibility of the nutrients.

There was no effect of treatments on fluid phase dynamics of the gastrointestinal tract (Table 4), with dilution rates of 10.05 $L.h^{-1}$ and recycling rate of 10.14 h.

Adding ground soybean grains to buffalo diets increased allantoin concentration in urine (P<0.05); this effect reflected an increase in microbial production and efficiency of microbial protein synthesis (Table 5). The effect was potentiated by the addition of YM and by combined antioxidants (YM and vitamin E).

Table 3 - Intake (kg/day), ruminal, intestinal and total digestibility of experimental diets offered to buffaloes

	Diet[1]				SEM	P[2]		
	Control	GS	GSYM	GSYME		1	2	3
				Dry matter				
Intake	7.58	7.20	7.23	7.06	0.15	<0.01	0.49	0.07
Ruminal digestibility	0.61	0.61	0.61	0.61	0.21	0.84	0.58	1.00
Intestinal digestibility	0.18	0.17	0.18	0.19	0.12	0.37	0.20	0.09
Total digestibility	0.66	0.66	0.68	0.67	0.14	0.33	0.26	0.36
				Organic matter				
Intake	6.53	6.25	6.23	6.18	0.14	<0.01	0.47	0.54
Ruminal digestibility	0.63	0.63	0.65	0.64	0.21	0.34	0.27	0.37
Intestinal digestibility	0.16	0.15	0.15	0.16	0.11	0.37	0.20	0.09
Total digestibility	0.69	0.69	0.71	0.70	0.13	0.33	0.26	0.36
				Crude protein				
Intake	0.76	0.73	0.74	0.72	0.21	0.04	0.78	0.09
Ruminal digestibility	0.22	0.24	0.23	0.21	0.12	0.64	0.13	0.26
Intestinal digestibility	0.66	0.66	0.68	0.67	0.14	0.34	0.27	0.37
Total digestibility	0.73	0.73	0.75	0.74	0.24	0.33	0.26	0.36
				Ether extract				
Intake	0.15	0.36	0.35	0.37	0.12	<0.01	0.34	0.09
Ruminal digestibility	0.051	0.052	0.050	0.052	0.08	0.41	0.39	0.16
Intestinal digestibility	0.86	0.86	0.86	0.86	0.12	0.84	0.58	1.00
Total digestibility	0.86	0.86	0.88	0.87	0.14	0.33	0.26	0.36
				Neutral detergent fiber				
Intake	3.83	3.61	3.82	3.54	0.23	0.01	0.22	<0.01
Ruminal digestibility	0.62	0.58	0.58	0.59	0.15	<0.01	0.25	0.08
Intestinal digestibility	0.08	0.08	0.08	0.08	0.11	0.41	0.39	0.16
Total digestibility	0.63	0.63	0.65	0.64	0.13	0.33	0.26	0.36
				Acid detergent fiber				
Intake	2.10	1.95	1.96	1.99	0.22	0.01	0.65	0.58
Ruminal digestibility	0.47	0.47	0.46	0.48	0.12	0.60	0.63	0.10
Intestinal digestibility	0.062	0.062	0.060	0.062	0.14	0.41	0.39	0.16
Total digestibility	0.49	0.50	0.51	0.50	0.11	0.33	0.26	0.36
				Total carbohydrates				
Intake	6.43	6.34	6.33	6.29	0.22	0.15	0.96	0.30
Ruminal digestibility	0.60	0.63	0.63	0.63	0.11	<0.01	0.61	0.19
Intestinal digestibility	0.097	0.097	0.095	0.097	0.11	0.41	0.39	0.16
Total digestibility	0.62	0.62	0.64	0.63	0.14	0.33	0.26	0.36
				Total digestible nutrients				
Intake	4.45	4.66	4.68	4.68	2.13	0.06	0.53	0.43

SEM - standard error of the mean.
[1] GS - diet with ground soybean grains; GSYM - diet with ground soybean grains supplemented with yerba mate; GSYME - diet with ground soybean grains supplemented with yerba mate and vitamin E.
[2] Contrast 1 = effect of soybean grain (control versus GS, GSYM, and GSYME); 2 = effect of yerba mate (GS versus GSYM and GSYME); 3 = effect of the antioxidant association (yerba mate + vitamin E) (GSYM versus GSYME).

Table 4 - Dynamics of the liquid phase in buffaloes fed the experimental diets

	Diet[1]				SEM	P[2]		
	Control	GS	GSYM	GSYME		1	2	3
Passage rate (L/h)	9.57	10.52	9.90	10.24	0.36	0.37	0.56	0.70
Ruminal volume (L)	87.49	92.64	85.60	90.89	1.99	0.68	0.45	0.43
Recycling time (h)	10.64	9.85	10.24	9.84	0.37	0.39	0.81	0.66
Flow rate (L/h)	8.46	9.89	8.52	9.33	0.49	0.46	0.39	0.53
Recycling time (times/day)	2.30	2.53	2.38	2.46	0.08	0.37	0.56	0.70

SEM - standard error of the mean.
[1] GS - diet with ground soybean grains; GSYM - diet with ground soybean grains supplemented with yerba mate; GSYME - diet with ground soybean grains supplemented with yerba mate and vitamin E.
[2] Contrast 1 = effect of soybean grain (control versus GS, GSYM, and GSYME); 2 = effect of yerba mate (GS versus GSYM and GSYME); 3 = effect of the antioxidant association (yerba mate + vitamin E) (GSYM versus GSYME).

Table 5 - Efficiency of microbial protein synthesis of buffaloes fed the experimental diets

	Diet[1]				SEM	P[2]		
	Control	GS	GSYM	GSYME		1	2	3
Urinary volume (L/day)	19.47	19.43	19.23	20.11	0.39	0.88	0.79	0.42
Uric acid (mmol/day)	0.143	0.200	0.227	0.194	0.01	0.66	0.60	0.18
Allantoin (mmol/day)	120.46	134.34	140.73	169.66	8.12	<0.01	<0.01	<0.01
Purine derivatives (mmol/day)	120.60	134.54	140.96	169.85	8.12	<0.01	<0.01	<0.01
Absorbed purines (mmol/day)	101.18	111.51	116.23	137.63	6.01	<0.01	<0.01	<0.01
Microbial N (g/day)	73.56	81.07	84.50	100.06	4.37	<0.01	<0.01	<0.01
Microbial crude protein (g/day)	459.80	506.70	528.10	625.40	7.34	<0.01	<0.01	<0.01
DOMR (kg)	4.13	3.96	4.16	4.06	0.02	<0.01	0.08	0.01
EMPS/DOMR (g microbial CP/kg DOMR)	111.32	127.95	126.95	154.03	4.51	<0.01	0.03	0.01

DOMR - digestible organic matter fermented in the rumen; EMPS - efficiency of microbial protein synthesis; CP - crude protein; SEM - standard error of the mean.
[1] GS - diet with ground soybean grains; GSYM - diet with ground soybean grains supplemented with yerba mate; GSYME - diet with ground soybean grains supplemented with yerba mate and vitamin E.
[2] Contrast 1 = effect of soybean grain (control versus GS, GSYM, and GSYME); 2 = effect of yerba mate (GS versus GSYM and GSYME); 3 = effect of the antioxidant association (yerba mate + vitamin E) (GSYM versus GSYME).

Table 6 - Mean values of concentration of short chain fatty acids (mmol/100 mL), pH, ammonia nitrogen ($N\text{-}NH_3$), acetate:propionate ratio in the rumen of buffaloes fed the experimental diets

	Diet[1]				SEM	P[1]		
	Control	GS	GSYM	GSYME		1	2	3
Acetate	60.68	61.49	60.00	60.23	0.16	0.46	<0.01	0.21
Propionate	15.53	15.38	15.73	15.88	0.08	0.35	0.02	0.42
Isobutirate	0.81	0.70	0.61	0.99	0.08	0.73	0.50	0.06
Butirate	8.48	9.16	8.94	9.06	0.09	<0.01	0.31	0.47
Isovalerate	1.63	1.53	1.37	1.69	0.07	0.50	0.97	0.10
Valerate	1.08	1.02	0.92	1.35	0.08	0.92	0.45	0.04
Acetate:propionate	3.91	4.00	3.81	3.79	0.02	0.13	<0.01	0.54
pH	6.58	6.65	6.54	6.57	0.44	0.52	0.51	0.55
$N\text{-}NH_3$ (mg/100 mL)	12.48	12.77	11.66	11.38	0.71	0.33	0.34	0.31

SEM - standard error of the mean.
[1] GS - diet with ground soybean grains; GSYM - diet with ground soybean grains supplemented with yerba mate; GSYME - diet with ground soybean grains supplemented with yerba mate and vitamin E.
[2] Contrast 1 = effect of soybean grain (control versus GS, GSYM, and GSYME); 2 = effect of yerba mate (GS versus GSYM and GSYME); 3 = effect of the antioxidant association (yerba mate + vitamin E) (GSYM versus GSYME).

Soybean grain feed increased the butyrate concentration, and addition of YM influenced the concentration of the main SCFA in the rumen, with a decrease in acetate concentration and an increase in propionate concentration, resulting in a lower acetate:propionate ratio (Table 6). Combining antioxidants resulted in increases of the concentration of branched-chain fatty acids, such as valerate (P = 0.04) and isobutyrate (P = 0.06), which are carbon skeleton sources for cellulolytic bacteria growth.

Experimental diets did not affect the pH and ammonia nitrogen concentration in the rumen. Ruminal pH showed quadratic behavior (pH = 0.0124.pH2 − 0.11113.pH + 6.7329; R^2 = 0.8888) as a function of collection time, with an estimated minimum pH of 6.45 at 3.48 h after feeding. Observed values of $N\text{-}NH_3$ concentration also showed quadratic behavior ($N\text{-}NH_3 = 0.4766.x^2 + 3.6605.x + 8.8706$; R^2 = 0.7351), with a maximum concentration of 17.98 mmol/L at 3.58 h after feeding.

Discussion

Adding soybean grain to diets of buffaloes increased EE intake, but decreased the intake of DM and other nutrients (OM, CP, NDF, and ADF; Table 3). This was likely due to the energy demands of animals that were met, which is supported by the observed intake of TDN that did not differ between experimental diets, indicating that energy requirements were met even with a reduction in DM intake caused by the soybean addition. This conclusion is supported by the theory of intake regulation by dietary energy (Van Soest, 1994).

A negative effect of soybean grain was observed only on ruminal digestibility of NDF and TC, with no effect on the total digestibility of these nutrients (Table 3). Polyunsaturated fatty acids (PUFA) present in soybean may coat the fiber when liberated in the rumen, reducing microbial colonization in the substrate (Gibb et al., 2005) and, as a consequence, reducing the rumen digestibility of fiber. Another factor that may contribute to reducing ruminal digestibility of NDF is the toxicity of PUFA to cellulolytic microorganisms in the rumen, which occurs due to changes in the permeability and integrity of their membranes (Jenkins, 1993). However, one way to revert the lipid action on ruminal microorganisms, according to Vázquez-Añón and Jenkins (2007), would be to add antioxidants to the diets. Vázquez-Añón and Jenkins (2007) suggested that oxidative stress resulting from the peroxides formed from added fats could affect microbial growth in the rumen. These authors observed that when adding a mixture of commercial antioxidants into diets containing oxidized fat or not, there was an increase in the digestibility of NDF, ADF, and TC. In the present study, there was a tendency to improve the ruminal digestibility of NDF (P = 0.08) and ADF (P = 0.10) with supplementation of both YM and vitamin E in diets. Agustinho (2017) verified that total digestibility of NDF was reduced by adding flaxseed oil to the diet, and digestibility was increased with vitamin E supplementation in diets offered to lactating buffaloes; however, no interaction between the addition of vitamin E and flaxseed oil in diets was observed on NDF digestibility. Naziroglu et al. (2002) also reported the importance of vitamin E action in ruminal metabolism by protecting the cellular membranes against the action of free radicals.

Our observed absence of effects from ground soybean on ruminal and total digestibility of DM, OM, CP, EE, and ADF is partially in agreement with Oliveira et al. (2007), who also observed no effect of different lipid sources, such as soybean grain, on nutrient digestibility of diets offered to lactating buffaloes. When oilseeds are used for lipid supplementation, the oil is released more slowly during the grain degradation process, and effects on the rumen are less pronounced. Thus, adding soybean to diets had no effect on ruminal digestibility of nutrients; however, it was enough to reduce ruminal digestibility of NDF, probably due to greater susceptibility of cellulolytic microorganisms to PUFA. It is noteworthy that EE content in the experimental diet (53.3 g/kg DM) was below the limit recommended for ruminants (70 g/kg DM) by Mir et al. (2001). Yerba mate supplementation did not interfere with ruminal and total nutrient digestibility. Similarly, Santos et al. (2017) also did not observe an effect of increasing levels of YM in diets containing canola seed on nutrient digestibility when given to lactating cows.

Passage rate of liquid phase is directly related to synthesis of microbial protein (Silva et al., 2011). However, in this study, although there were no changes in the dynamics of liquid phase (Table 4), an effect on the synthesis of microbial protein resulting from ground soybean, YM, and the combination

of YM and vitamin E was observed (Table 5). Isaacson et al. (1975) also observed that microbial protein synthesis per fermentable carbohydrate unit increased with high liquid-phase passage rate.

We observed a reduction in the DOMR, but no effect on the concentration of N-NH_3 in the rumen; however, there was an increase in the synthesis of microbial protein, due to the addition of soybean ground and antioxidants (YM and vitamin E). Thus, although protozoan population has not been evaluated in this study, the literature suggests that the observed increase in microbial production is due to the action of lipids and antioxidants on the microbial population.

The increased production of microbial nitrogen observed with ground soybean addition in diets (Table 5) suggests that synthesis of microbial protein can be influenced indirectly by lipids, by the decrease in protozoa population (Dewhurst et al., 2000). Reduction of protozoa population in the rumen also decreases N recycling and increases the amount of microbial N that of the intestines (Koenig et al., 2000; Paula et al., 2016).

Effects of YM on the synthesis of microbial protein may have occurred due to saponins present in YM, which reduces the population of ciliate protozoa in the rumen (Castro-Montoya et al., 2011). Thus, the effect of saponin in the rumen seems to be similar to that of lipids, as mentioned above. Saponin acts on protozoa by forming irreversible saponin complexes with cholesterol from the cell membranes of these microorganisms (Williams and Coleman, 1997). Using both YM and vitamin E combined the protective action of vitamin E on cellular membranes with the action of antioxidants from YM, which resulted in increased production and efficiency of ruminal microbial synthesis when compared with YM alone. Similarly, an associative effect of antioxidants in the diet was observed by Hino and Kuroda (1993) on microbial production in the rumen. Vázquez-Añón and Jenkins (2007) reported that the synthesis of microbial protein was reduced when using oxidized fat, and this effect was neutralized with the addition of a mixture of antioxidants (liquid blend of ethoxyquin and tertiary butyl hydroquinone).

Experimental diets did not influence pH and ammonia nitrogen concentration in the rumen but did influence the concentrations of SCFA (Table 6). Addition of soybean to the diets increased butyrate concentration, which is a positive result because butyrate is converted to B-OH-butyrate (Van Soest, 1994) and becomes fully available as an energy source for peripheral tissues.

Effects of antioxidants were observed on rumen SCFA concentrations (Table 6). Yerba mate increased the propionate concentration and reduced the acetate concentration, decreasing the acetate:propionate ratio, which suggests an increase in energy available to ruminant. In addition, considering that synthesis of acetate occurs in parallel with the format, a precursor methane, the decrease of acetate may have reduced methane synthesis.

Effects of combined antioxidants, YM and vitamin E, were observed on the branched-chain fatty acids, such as valerate, which increased in concentration. A tendency toward increased ($P = 0.06$) isobutyrate concentration was also observed. These branched-chain fatty acids are specific sources of ketoacids and are necessary for protein synthesis of many rumen microorganisms, especially the main cellulolytics, which do not synthesize them. An increase in branched-chain fatty acids, which are a growth factor for fibrolytic microorganisms, during ruminal fermentation with antioxidant addition had a positive effect on the rumen degradation process.

Conclusions

Using lipids in the grain form in buffalo diet has positive effects on ruminal metabolism without affecting total digestibility.

Supplementing diets containing ground soybeans with the antioxidants yerba mate and vitamin E improves the production and efficiency of microbial protein synthesis.

Positive effects of antioxidants (yerba mate and vitamin E) in diets containing ground soybeans are confirmed not only on the ruminal microbiota but also on ruminal fermentation products of buffaloes (acetate, propionate, and butyrate).

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