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Short Communication

In vitro fermentation characteristics of ruminant diets using ethanol extract of brown propolis as a nutritional additive

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ABSTRACT - The addition of levels of ethanol extract of brown propolis was evaluated by assessing diet degradation in rumen fluid and predicting cumulative *in vitro* gas production by nonlinear (dual pool logistic and exponential) models. A total of 35 g of crude propolis were extracted in 65 mL of cereal alcohol (95% ethanol). In a completely randomized factorial design, the experimental diets combined four concentrations of extracted propolis diluted in cereal alcohol (0, 50, 70, and 100% of propolis extract) and supplementation doses (4, 8, 12, 16, and 20 mL/kg dry matter), tested in triplicate. Diet (400 g/kg Tifton hay and 600 g/kg concentrate) was incubated for 96 h carried out three times in three different weeks. There was significant interaction between extract concentration and dose on the dry matter (DM) degradability. Dry matter degradability of diet decreased exponentially as a function of the increase in dose ($y = 678.55 \times dose^{-0.271}$). Pure alcohol treatment showed a negative exponential effect, with degradability of 303.61 g/kg when administered at a dose of 20 mL/kg DM. Treatment 100% ethanol extract reached the greatest degradability, estimated at 18.93 mL/kg DM. The treatment with 70% extract showed 6.35 mL/kg DM and the 50% extract, 7.65 mL/kg DM of minimum degradability. The reduction potential of pure ethanol was –0.32 mL gas/mL. Estimates of maximum gas production by dual pool logistic and exponential models were 13.10 mL and 12.07 mL for 100% extract, respectively. The 100% extract produced the highest gas production estimates, above 30 mL gas/100 mg DM of fermented diet. The degradation and fermentation of ruminant diet can be improved using 13 mL/DM kg of ethanol extract of propolis.

Key Words: feed additive, gas production, propolis, ruminal degradability, ruminant nutrition

Introduction

Propolis is a natural product with antimicrobial activity (Park et al., 2000; Stradiotti Júnior et al., 2004b). The chemical composition of propolis is quite complex and diversified because it depends on the ecology of plants visited by bees that produce it (Ghisalberti, 1979). Several studies have demonstrated that the antimicrobial activity of propolis occurs by the inhibition of bacteria classified as Gram-positive (Ghisalberti, 1979; Bankova et al., 2000; Vargas et al., 2004). However, the effects of dilution according to the type of propolis still have to be elucidated to obtain solutions with sufficient active principle to obtain such effects on the rumen microbiota.

According to Mirzoeva et al. (1997), propolis and some of its components, such as caffeic acid phenethyl ester and quercetin, are bacteriostatic to Gram-positive and some Gram-negative bacteria, inhibiting their motility, likely because they modify the bionergenic status of bacterial membranes. This action is similar to that of ionophores, which are commonly included in ruminant diet because of their conditioning role in the ruminal environment, capable of improving the utilization of metabolic energy and decreasing lactate levels and protein deamination (Prado et al., 2010).

Like ionophores, propolis has been used as an additive in ruminant nutrition to inhibit the production of gases, particularly methane, and to decrease nitrogen losses during ruminal fermentation (Stradiotti Júnior et al, 2001; Stradiotti Júnior et al., 2004a; Ítavo et al., 2011; Heimbach et al., 2014). Silva et al. (2014) studied the effects of dietary

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brown propolis on nutrient intake and digestibility in feedlot lambs compared with monensin and concluded that the addition of brown propolis has the same effect as monensin, with neither of them maximizing nutrient availability in diets for feedlot lambs at seven months of age.

According to Makkar (2005), *in vitro* gas production has been considered a suitable method to assess the action of phytochemicals on ruminal microbial fermentation. Groot et al. (1996) reported that different nonlinear models with specific assumptions and parameters are available to fit curves of cumulative *in vitro* gas production, allowing degradation parameters to be determined and increasing understanding of fermentation kinetics.

The present study tested the addition of different concentrations and doses of brown propolis extract to ruminant diet and evaluated the effects of this supplementation on diet degradation in rumen fluid, in addition to assessing the kinetics of cumulative *in vitro* gas production through exponential (Ørskov and McDonald, 1979) and dual pool logistic (Schofield et al., 1994) nonlinear models.

Material and Methods

The experiment was carried out in Campo Grande, Mato Grosso do Sul, Brazil. The protocols adopted were approved by the local Animal Research Ethics Committee (case no. 218/2009).

Crude brown propolis was collected from *Apis mellifera* hives in an apiary located in Terenos, Mato Grosso do Sul (20º26'34.31" S, 54º50'27.86" W; 530.7 m altitude). For propolis production, a nylon mesh screen was placed between the hive body and the cover. After 45 days, the screens were removed, packed, and transported to the laboratory in Campo Grande. The propolis was produced from flowering plants in the area, mostly *Vernonia* spp. and *Cecropia pachystachya*, as well as *Luehea* sp., *Piptadenia falcata*, *Tabebuia* spp., and *Tabebuia caraiba*.

Propolis extract was obtained with 35 g of crude propolis extracted in 65 mL of cereal alcohol (ethanol, 95% NBR 5991). The extraction lasted 45 days, with daily stirring, and after the stock solution was filtered in filter paper, it was stored in an amber flask at room temperature. As determined by current Brazilian legislation (Brasil, 2001), the extract underwent physicochemical analysis for determination of waxes, dry residue, total phenols, and total flavonoids, as described by Funari and Ferro (2006). Ethanol propolis extract exhibited 29.90 mg/mL wax, 151.28 mg/mL dry residue, 27.65 mg/mL total phenols, and 13.98 mg/mL total flavonoids (Table 1).

Different concentrations of propolis extract were added to a diet containing 40:60 roughage:concentrate ratio (in dry matter basis), using Tifton grass as roughage and corn, soybean meal, and mineral supplement as concentrate.

The following concentrations of propolis extract were tested: no extract (negative control with 0% propolis extract and 100% grain ethanol); 50% extract (50% propolis extract + 50% water); 70% extract (70% propolis extract + 30% water); 100% extract (100% propolis extract).

The extract was added to the concentrate fraction of the diet and this mixture was added to the hay (Table 2). Four diets were administered at doses of 4, 8, 12, 16, and 20 mL/kg of dry matter (DM).

Diet samples were pre-dried in a forced-ventilation oven at 55 ºC for 72 h and ground in a mill equipped with 1-mm sieve mesh. Dry matter, organic matter, crude protein, and ether extract were determined according to AOAC methods 930.15, 932.05, 976.05, and 920.39, respectively (AOAC, 2000), and neutral detergent fiber (NDF) and acid detergent fiber were determined without sulfite and thermostable amylase, following the protocol described by Goering and Van Soest (1970). Non-fiber carbohydrates (NFC) were obtained by the equation proposed by Sniffen et al. (1992), in which NFC = total carbohydrates – NDF (Table 2).

Table 1 - Composition of ethanol extract of brown propolis

	Ethanol extract of brown propolis[1]
Wax (mg/mL)	29.90
Dry residue (mg/mL)	151.28
Total phenol (mg/mL)	27.65
Total flavonoid (mg/mL)	13.98

[1] A total of 35 g of crude propolis extracted in 65 mL of cereal alcohol (ethanol, 95% NBR 5991) over 45 days.

Table 2 - Diet composition

Ingredient	Content
Ground corn (g/kg)	435.00
Soybean meal (g/kg)	138.00
Mineral supplement (g/kg)[1]	17.00
Urea (g/kg)	10.00
Tifton hay (g/kg)	400.00
Proximate composition	
Dry matter (DM; g/kg)	915.29
Organic matter (g/kg DM)	921.20
Crude protein (g/kg DM)	180.54
Ether extract (g/kg DM)	27.78
Neutral detergent fiber (g/kg DM)	429.99
Acid detergent fiber (g/kg DM)	202.27
Non-fiber carbohydrate (g/kg DM)	28.30
Metabolizable energy[2] (Mcal/kg DM)	1.92

[1] Content per kilogram: 153 g Ca, 90 g P, 50 g S, 72g Na, 20 mg Co, 250 mg Cu, 900 mg F, 28 mg I, 600 mg Mn, 9 mg Se, 1800 mg Zn.
[2] Estimate on metabolizable energy: total digestible nutrients × 0.82.

To determine cumulative *in vitro* gas production, 0.5-g portions of the diet were sampled in triplicate and incubated with artificial saliva (Marten and Barnes, 1980) and inoculum obtained from two fistulated cows, pasture-fed, and provided with protein-energy supplement as described by Campos et al. (2000). Digestion kinetics were carried out three times for 96 h in three different weeks by assessing gas production from diet fermentation, recorded by a wireless system with radio frequency pressure transducer (Ankom® RF - Gas production system). Data on pressure (in psi) were recorded every 10 min and converted to mL of gas/100 mg DM of the fermented diet sample.

Cumulative gas production was predicted for each fraction using the following nonlinear models:

Dual pool logistic model (Schofield et al., 1994):

$$y = A/\{1+\exp^{[2+4.B.(C-t)]}\}+D/\{1+\exp^{[2+4.E.(C-t)]}\},$$

in which y = the volume of gas produced at time t; A = the volume of gas (mL) produced from the very rapidly degradable fraction (soluble sugar, amide, soluble amino acid, and non-protein nitrogen); B = degradation rate of the rapidly degradable fraction; C = lag time (h) for bacterial colonization and fermentation onset; D = volume of gas (mL) produced from the more slowly degradable fraction (cellulose, hemicellulose, and true protein); and E = rate of degradation of the slowly degradable fraction.

Exponential model (Ørskov and McDonald, 1979):

$$y = a+b.(1- \exp^{-k.t}),$$

in which y = the gas produced at time t; a = the volume of gas (mL) produced from the very rapidly degradable fraction (soluble sugar, amide, soluble amino acid, and non-protein nitrogen); b = the volume of gas (mL) produced from the potentially degradable fraction (fiber and protein); k = degradation rate of fraction b; and t = incubation time.

The Gauss-Newton algorithm, an iterative method of the non-linear regression tool (NLIN procedure) of SAS (Statistical Analysis System, version 9.0), was used to estimate the parameters of the models. Parameter estimates were subjected to analysis of variance and regression as a function of propolis extract concentration and dose. Significance was declared at $P<0.05$.

In a completely randomized 4 × 5 factorial design, combining four propolis extract concentrations (0, 50, 70, and 100%) and five supplementation doses (4, 8, 12, 16, and 20 mL/kg of diet DM), the diets subjected, in triplicate, to 96 h of *in vitro* fermentation were evaluated according to the following statistical model:

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + \alpha\times\beta_k + P_l + \varepsilon_{ijklm},$$

in which μ = is the overall mean; α_i = effect of extract concentration i, i = 1,..., 4; β_j = effect of dose j = 1, ..., 5; $\alpha\times\beta_k$ = is the interaction effect of extract concentration and dose; P_l = is the effect of period l = 1, …, 3; and ε_{ijklm} = is the experimental error of each Y_{ijklm} observation. All random effects were considered ~N (0, σ2e). Significance was declared at $P<0.05$.

Results

The interaction effect of propolis extract concentration and dose on DM degradability was significant (Table 3). In the treatment without propolis extract (negative control with pure ethanol only), DM degradability was 678.55 g/kg and it decreased exponentially as a function of the increase in dose ($y = 678.55\times dose^{-0.271}$; Table 2), obtaining the lowest value (303.61 g/kg) with a pure ethanol dose of 20 mL/kg DM. On the other hand, the use of 100% extract resulted in the highest *in vitro* degradability (Table 3), estimated at 18.93 mL/kg DM.

Diets testing the 70% extract showed minimum degradability estimates with supplementation of 6.35 mL/kg DM and for 50% extract, it was 7.65 mL/kg DM.

As estimated by both the dual pool logistic and exponential models, extract concentration and dose also affected cumulative gas production (Table 4). The negative control without propolis (pure ethanol) decreased gas production. The reduction potential estimated by the dual pool logistic model was –0.32 mL of gas per milliliter of ethanol added.

The diets added with propolis extract exhibited quadratic behavior as a function of supplementation dose (Table 4), except for 50% extract, which increased cumulative *in vitro* gas production linearly according to the exponential model ($Y_{exponential} = 14.4549 + 0.0576799.dose$; $R^2 = 0.94$).

The dual pool logistic model predicted that maximum cumulative *in vitro* gas production using 70% extract is achieved with a dose of 11.43 mL ($Y = 13.2401 + 1.90770.dose – 0.0834357.dose^2$; $R^2 = 0.92$). Maximum gas production using 100% extract would be obtained with a dose of 13.10 mL ($Y = 16.5623 + 3.69375.dose – 0.140931.dose^2$; $R^2 = 0.92$). Likewise, the maximum estimates predicted by the exponential model were obtained using 12.60 mL of 70% extract and 12.07 mL of 100% extract, respectively (Table 4).

Discussion

Ethanol propolis extract showed contents of wax (29.90 mg/mL), dry residue (151.28 mg/mL), total phenols (27.65 mg/mL), and total flavonoids (13.98 mg/mL) above the quality parameters established by Brazilian law (IN.3, Brasil, 2001), which determines minimum levels of 0.25% flavonoids (2.5 mg/mL) and 0.50% phenolic compounds (5.0 mg/mL).

Table 3 - Dry matter degradability (g/kg DM) after 96 h *in vitro* diet fermentation, as a function of the concentration and dose of propolis extract addition

Dose (mL/kg DM)	Extract concentration				SEM	P	
	0%[1] (100% ethanol)	50%[2]	70%[3]	100%[4]		Linear	Quadratic
0	678.55	678.55	678.55	678.55	1.619	0.211	0.345
4[a]	466.94	522.20	592.87	686.23	2.629	0.015	0.201
8[b]	381.09	546.17	638.76	782.48	4.424	0.037	0.154
12[c]	347.06	654.04	677.09	927.22	5.716	0.010	0.201
16[d]	306.35	720.88	723.74	806.58	5.733	0.039	0.215
20[e]	303.61	724.67	794.07	872.81	6.072	0.007	0.232
SEM	2.130	1.123	0.975	1.774			
P linear	0.029	0.015	0.050	0.040			
P quadratic	0.185	0.015	0.050	0.040			

DM - dry matter; SEM - standard error of the mean.
[1] $y = 678.55 \times dose^{-0.271}$ ($R^2 = 0.98$)
[2] $y = 653.067 - 18.7350.dose + 1.22517.dose^2$ ($R^2 = 0.90$)
[3] $y = 667.682 - 11.8769.dose + 0.934635.dose^2$ ($R^2 = 0.90$)
[4] $y = 664.197 + 21.2811.dose - 0.561958.dose^2$ ($R^2 = 0.76$)
[a] $y = 447.918 + 2.16619.extract\_concentration$ ($R^2 = 0.92$)
[b] $y = 368.143 + 3.98147.extract\_concentration$ ($R^2 = 0.99$)
[c] $y = 345.124 + 5.56991.extract\_concentration$ ($R^2 = 0.97$)
[d] $y = 362.326 + 5.03752.extract\_concentration$ ($R^2 = 0.88$)
[e] $y = 353.486 + 5.82373.extract\_concentration$ ($R^2 = 0.93$)

Table 4 - Cumulative gas production (mL/100 mg DM) over 96 h *in vitro* fermentation, estimated by the dual pool logistic and exponential models as a function of the concentration and dose of propolis extract added to the diet

Dose (mL/kg DM)	Extract concentration				SEM	P	
	0% (100% ethanol)	50%	70%	100%		Linear	Quadratic
			Dual pool logistic model				
0	14.26	14.26	14.26	14.26	0.124	0.185	0.214
4[a]	10.66	14.01	16.19	35.13	0.369	0.001	0.345
8[b]	9.52	13.17	23.76	35.60	0.381	0.001	0.312
12[c]	9.59	13.22	27.07	39.92	0.322	0.001	0.354
16[d]	7.66	15.81	20.43	36.79	0.337	0.001	0.365
20[e]	6.90	16.19	18.26	36.09	0.315	0.001	0.451
SEM	0.043	0.023	0.082	0.176			
P linear	0.001	0.001	0.001	0.001			
P quadratic	0.156	0.001	0.001	0.001			
			Exponential model				
0	14.24	14.24	14.24	14.24	0.615	0.196	0.255
4[f]	10.85	15.20	16.85	37.16	0.0322	0.001	0.365
8[g]	9.56	15.17	24.19	38.12	0.303	0.001	0.345
12[h]	8.47	15.27	22.78	36.29	0.321	0.001	0.289
16[i]	8.63	14.48	19.12	37.56	0.309	0.001	0.312
20[j]	7.86	16.05	20.95	31.93	0.302	0.001	0.365
SEM	0.365	0.324	0.322	0.386			
P Linear	0.001	0.002	0.001	0.001			
P Quadratic	0.001	0.378	0.365	0.001			

DM - dry matter; SEM - standard error of the mean.
$Y_{0\%\ dual\text{-}pool} = 12.9597 - 0.321742.dose$ ($R^2 = 0.93$)
$Y_{50\%\ dual\text{-}pool} = 14.5422 - 0.29840.dose + 0.0199332.dose^2$ ($R^2 = 0.82$)
$Y_{70\%\ dual\text{-}pool} = 13.2401 + 1.90770.dose - 0.0834357.dose^2$ ($R^2 = 0.92$)
$Y_{100\%\ dual\text{-}pool} = 16.5623 + 3.69375.dose - 0.140931.dose^2$ ($R^2 = 0.92$)
[a] $Y_{4mL/kg\ DM} = 3.99034 + 0.251111.extract\_concentration$ ($R^2 = 0.82$)
[b] $Y_{8\ mL/kg\ DM} = 7.55918 + 0.229868.extract\_concentration$ ($R^2 = 0.90$)
[c] $Y_{12\ mL/kg\ DM} = 8.8314 + 0.145093.extract\_concentration$ ($R^2 = 0.67$)
[d] $Y_{16\ mL/kg\ DM} = 4.12625 + 0.257379.extract\_concentration$ ($R^2 = 0.82$)
[e] $Y_{20\ mL/kg\ DM} = 11.5382 + 0.102519.extract\_concentration$ ($R^2 = 0.93$)
$Y_{0\%\ exponential} = 13.2719 - 0.317609.dose$ ($R^2 = 0.85$)
$Y_{50\%\ exponential} = 14.4549 + 0.0576799.dose$ ($R^2 = 0.94$)
$Y_{70\%\ exponential} = 14.1354 + 1.33265.dose - 0.05290.dose^2$ ($R^2 = 0.78$)
$Y_{100\%\ exponential} = 16.2216 + 4.07810.dose - 0.16892.dose^2$ ($R^2 = 0.88$)
[f] $Y_{4\ mL/kg\ DM} = 6.20293 + 0.246591.extract\_concentration$ ($R^2 = 0.75$)
[g] $Y_{8\ mL/kg\ DM} = 6.43568 + 0.278582.extract\_concentration$ ($R^2 = 0.88$)
[h] $Y_{12\ mL/kg\ DM} = 8.67237 + 0.202814.extract\_concentration$ ($R^2 = 0.92$)
[i] $Y_{16\ mL/kg\ DM} = 5.10757 + 0.26826.extract\_concentration$ ($R^2 = 0.82$)
[j] $Y_{20\ mL/kg\ DM} = 10.0372 + 0.1870.extract\_concentration$ ($R^2 = 0.85$)

Dry matter degradability decreased exponentially as a function of the increase in dose (Table 3). Thus, including increasing doses of pure ethanol in the rumen fluid has a negative effect on microbial activity and substrate fermentation by anaerobic microorganisms in the rumen fluid. Likewise, the negative control also decreased cumulative *in vitro* gas production, yielding 6.9 and 7.9 mL/100 mg DM according to the dual pool logistic and exponential models, respectively (Table 4).

The use of 100% extract resulted in the highest *in vitro* degradability (Table 3), which suggests that components in the propolis extract promoted increasing degradation of diet DM in the rumen fluid, likely through the selection and stimulation of certain rumen bacteria, especially the Gram-negative variety.

The antimicrobial action of propolis on bacterial growth, membrane potential, and motility was studied by Mirzoeva et al. (1997). They found that propolis affects the permeability of the bacterial inner membrane to ions and causes dissipation of membrane potential, hindering ATP synthesis, ion transport, and motility of Gram-positive bacteria.

The antibacterial activity of propolis against Gram-positive bacteria is strong but limited against Gram-negative bacteria (Bankova et al., 1999; Marcucci et al., 2001; Packer and Luz, 2007). Although the cell walls of Gram-negative bacteria are less rigid than those of their Gram-positive counterparts, their higher resistance to propolis likely results from the higher complexity of these structures, with liposaccharides and high lipid content (Vargas et al., 2004).

The flavonoids contained in the propolis extract act against microorganisms through inhibition of cell membrane function, bacterial activity, or synthesis of nucleic acid (Cushnie and Lamb, 2005). This explains the higher degradability and cumulative gas production of diets (Tables 3 and 4) added with propolis extract in relation to the negative control, which had complete bactericidal action.

The treatment with 70 and 50% extract showed minimum degradability estimates close to 7 mL/kg DM (6.35 and 7.65 mL/kg DM, respectively). These similar estimates indicates that even after extract dilution in 30 or 50% water, microbial fermentation and gas production are still affected (Tables 3 and 4). The results suggest that the content of 13.98 mg/mL flavonoids in the extract was probably capable of affecting fermentation in rumen fluid, acting through bacteria selection.

The dilution of propolis ethanol extract in water reduced its bacteriostatic action, given that it lowers the content of active compounds in the diet. Propolis flavonoids, such as galangine, quercetin, pinocembrin, and kaempferol, are natural polyphenolic compounds widely spread among seed plants. Propolis also contains aromatic acids and esters, aldehydes and ketones, terpenoids and phenylpropanoids (such as caffeic and chlorogenic acids), esteroids, amino acids, polysaccharides, hydrocarbons, fatty acids, and low amounts of a number of compounds (Bankova et al., 2000; Packer and Luz, 2007; Lustosa et al., 2008), which are considered as total phenols (27.65 mg/mL) in the analysis.

Park et al. (1998) found that flavonoids are mostly extracted in ethanol solutions at 60 to 80% concentration, which inhibits microbial growth satisfactorily. They also report that ethanol extracts at 70 to 80% show significant antioxidant activity, similar to that observed with 100% extract in the present study (Tables 2 and 3), in addition to being beneficial to ruminal diet degradability and *in vitro* gas production.

Oliveira et al. (2004) studied the effects of monensin and propolis extract on *in vitro* degradability of crude protein from different nitrogen sources using ruminal fluid from cattle grazing *Brachiaria* spp. grass. They found that both monensin and propolis extract reduced the production of ammonia from highly degradable protein sources; however, propolis was better because it reduced deamination (Stradiotti Júnior et al., 2001), which can increase microbial activity and efficiency, given that rumen bacteria optimize the use of dietary nitrogen sources. This corroborates with the present study, in which *in vitro* degradability was higher in diets added with propolis extract (Table 2).

As estimated by both dual pool logistic and exponential models, extract concentration and dose also affected cumulative gas production (Tables 3 and 4). The negative control without propolis (pure ethanol) decreased gas production, likely because of its bactericidal action, which eliminated rumen fluid microorganisms.

The reduction potential estimated by the dual pool logistic model was –0.32 mL of gas per milliliter of ethanol added. Similarly, the estimates provided by the exponential model indicated a negative effect of the negative control using pure ethanol, with similar reduction potential of –0.32 mL of gas per milliliter of ethanol. Alcohol acts on protein denaturation and lipid solubilization. There may be side effects on the interference of metabolism and eventual lysis of cells. Proteins can be denatured by extremes of pH and by certain miscible organic solvents such as alcohol (Nelson and Cox, 2012).

The result of cumulative *in vitro* gas production (Table 3) suggests that *in vitro* gas production increases with dose of propolis ethanol extract due to the higher dietary flavonoid and total phenol content.

The maximum cumulative *in vitro* gas production predicted by both models using 100% ethanol extract of propolis were obtained with doses of 13.10 mL and 12.07 mL, respectively. Ítavo et al. (2011) suggested the use of brown propolis extract for 15 mL/kg DM as a substitute for sodium monensin to improve feed conversion in confined lambs. In the present study, a positive effect was obtained using 13 mL/kg DM, which reinforces the importance of *in vitro* analysis given that the results produced may be economically beneficial in large-scale administration. In another work, Ítavo et al (2011) concluded that different levels of green propolis extract in the diet of feedlot lambs did not influence nutrient digestibility and recommended the inclusion of 7.60 mL (2.1189 mg of dry matter and 0.1123 mg of flavonoids) of green propolis extract/day in the diet of confined lambs to maximize efficiency,.

The findings indicate that dietary propolis improves DM degradation (Table 2), likely through bacterial selection by bacteriostatic action and cumulative gas production (Tables 3 and 4). However, the dose of extract needed to improve diet degradability is limited, as shown by the quadratic behavior of the estimates. This is probably related to the rumen environment; that is, the *in vitro* assay does not include factors such as passage rate and gradual extract dilution, which can impair the optimal action of propolis solutions as a diet additive because of their alcohol content.

The highest gas production estimates (above 30 mL gas/100 mg fermented DM) were obtained with the diet with 100% ethanol extract of propolis (Tables 3 and 4). In a study on the addition of residues from alcoholic extraction of brown propolis to a ruminant diet, Heimbach et al. (2014) reported 18.18 mL *in vitro* gas production using a dose of 10 g/kg DM and incubation in ruminal fluid of lambs. In bovine ruminal fluid, the highest gas production they reported is 16.89 mL, obtained with diet with residue inclusion of 5 g/kg DM. The diet tested also consisted of Tifton hay combined with corn and soybean meal-based concentrate, but using a 50:50 roughage:concentrate ratio.

The dose of 20 mL of 70% extract exhibited average gas production of 18.26 mL/100 mg DM (Tables 3 and 4), which is close to the value of 18.78 mL reported by Heimbach et al. (2014). The difference in gas production estimates using 70 and 100% extracts and the results found by those authors are likely related to the phenol and total flavonoid content in the extracts. The propolis extraction residue tested contained 0.24 mg of total phenols and 0.35 mg of total flavonoids per gram of dry residue, whereas 100% extract exhibited 151.28 mg/mL of dry residue, 27.65 mg/mL of phenols, and 13.98 mg/mL of total flavonoids. Given the higher phenol and flavonoid content in propolis extract compared with its residue, the higher effect of the former on ruminal fluid bacteria is expected, along with higher degradability and *in vitro* gas production. Thus, the diets added with 100% ethanol extract of brown propolis may lead to the greatest degradability rates and cumulative *in vitro* gas production.

Conclusions

The diets added with 100% ethanol extract of brown propolis prepared with 35 g of propolis and 65 mL of cereal alcohol promote the greatest diet degradability and cumulative *in vitro* gas production. Ethanol extract of brown propolis can be included as nutritional additive in ruminant diets. The maximum dose of 100% propolis extract supplementation recommended, which improves degradation and fermentation of ruminant diets, is 13 mL/kg DM.

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