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Residue of propolis extract in bovine diets with increasing levels of protein on rumen fermentation

Abstract – The objective of this work was to evaluate the effect of the residue from the extraction of propolis, added to bovine diets with increasing levels of protein, on ruminal fermentation in vitro. For this, the in vitro gas production technique was used. Incubation was carried out with inocula from three fistulated cows, in three periods. In each period, a cow received a daily dose of 100 g propolis residue. Four diets were evaluated: corn silage (control); and 25, 50, and 75% concentrate based on soybean meal. The following were determined: kinetics of rumen fermentation; dry matter degradation; production of gases, volatile fatty acids (acetate, propionate, and butyrate), methane, and ammonia nitrogen; and pH. The inclusion of 14.4, 15.1, and 9.5% propolis residue, respectively, to 25, 50, and 75% concentrate increased the production of gases from the degradation of fibrous carbohydrates, when compared with the control. The propolis residue reduces methane production and the acetate:propionate ratio at all tested concentrate inclusion levels.

Index terms: additive, ammoniacal nitrogen, methane, nutrition.

Resíduo do extrato de própolis em dietas de bovinos com níveis crescentes de proteína sobre a fermentação ruminal

Resumo – O objetivo deste trabalho foi avaliar o efeito do resíduo da extração de própolis, adicionado em dietas de bovinos com níveis crescentes de proteína, sobre a fermentação ruminal in vitro. Para tanto, utilizou-se a técnica de produção de gás in vitro. A incubação foi realizada com inóculos de três vacas fistuladas, em três períodos. Em cada período, uma vaca recebeu uma dose diária de 100 g do resíduo de própolis. Foram avaliadas quatro dietas: silagem de milho (controle); e 25, 50 e 75% de concentrado à base de farelo de soja. Foram determinados: cinética de fermentação ruminal; degradação da matéria seca; produção de gases, ácidos graxos voláteis (acetato, propionato e butirato), metano e nitrogênio amoniacal; e pH. A inclusão de 14,4, 15,1 e 9,5% de resíduo de própolis, respectivamente, a 25, 50 e 75% de concentrado aumentou a produção de gases oriundos da degradação dos carboidratos fibrosos, em comparação ao controle. O resíduo de própolis reduz a produção de metano e a relação acetato:propionato em todos os níveis de inclusão de concentrado testados.

Termos para indexação: aditivo, nitrogênio amoniacal, metano, nutrição.

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Introduction

The manipulation of the rumen environment aims to reduce energy losses and increase feed conversion, resulting in a better use of the diet

and, consequently, in a reduction in the production cost of cattle due to a lower expenditure on feed (Clemmons et al., 2019). This optimization is important since, daily, from 2 to 12% of the gross energy of feeds is lost by the production of methane (CH_4) (Patra, 2012).

CH_4 production occurs in the digestive tract of ruminants, eliminating the H_2 produced and maintaining fermentation; however, in this process, there is an energy loss of 9.45 kcal L^{-1} of produced CH_4 (Brouwer, 1965; Guan et al., 2006). Therefore, CH_4 production represents rumen inefficiency and its emission contributes to the greenhouse effect (Castillo-González et al., 2014).

Increasing efficiency, however, is possible by changing the composition of the diet and introducing additives such as ionophore substances with an antibiotic effect, allowing, for example, a greater production of propionate and a lower production of CH_4 due to a reduction in the population of Gram-positive bacteria (Nicodemo, 2001; Rangel et al., 2008; Tadesse, 2014). In production systems, mainly in feedlots, ionophores are among the most used primary additives (Oliveira & Millen, 2014).

Despite the cited benefits, ionophores have been identified as a potential risk to human health because they may cause bacterial resistance. However, antimicrobial additives such as these substances cannot be withdrawn abruptly due to the risk of a major negative impact on production systems (Hao et al., 2014). This scenario is complex and shows the need of finding alternative additives that can contribute to a greater feed conversion and a reduction in CH_4 production, without posing risks to human health.

The propolis produced by bees (*Apis mellifera* sp.) from a combination of plant exudates, wax, enzymes, and pollen, among other elements, may be an alternative antimicrobial additive mainly because of its antibacterial properties (Packer & Luz, 2007). Gram-positive bacteria are more sensitive to the action of propolis, as observed for ionophores (Aguiar et al., 2013). However, some authors have reported positive results for Gram-negative bacteria, such as the reduction in CH_4 production (Leopoldino et al., 2007; Ehtesham et al., 2018), the reduction in amino acid deamination, and the maintenance of ruminal ammonia (Stradiotti Júnior et al., 2004). It should be noted that, in these studies, alcoholic extracts of propolis were used, which would be unviable as an additive in animal feed, especially in diets for cattle, considering their high costs; however, the propolis residue generated after alcoholic extraction still presents active principles (Heimbach et al., 2016) and could be a viable, sustainable, and low-cost alternative to be added to cattle diets.

The objective of this work was to evaluate the effect of the residue from the extraction of propolis, added to bovine diets with increasing levels of protein, on ruminal fermentation in vitro.

Materials and Methods

The experiment was carried out at the José Henrique Bruschi experimental field, located at the livestock bioefficiency and sustainability experimental complex of Embrapa Gado de Leite, in the municipality of Coronel Pacheco, in the state of Minas Gerais, Brazil. All experimental procedures using animals were approved by Embrapa's ethics committee on animal use, protocol number 03/2014.

The used residue was obtained from a brown propolis extract purchased from Cooperativa Nacional de Apicultura, located in the municipality of Nova Lima, also in the state of Minas Gerais, Brazil.

Three Girolando rumen-fistulated cows were used as donors of the ruminal liquid for in vitro incubation. The cows were kept on a maintenance-level diet, consisting of corn silage and 2 kg concentrate made up of corn (*Zea mays* L.) meal, soybean [*Glycine max* (L.) Merr.], and a mineral-vitamin premix. Inoculum-donor cows were subjected to treatments with or without the addition of propolis residue during three experimental periods of 14 days. In each period, a different animal received 100 g propolis residue and, after 14 days of adaptation, the inocula was collected with and without propolis for incubation. The addition of the 100-g residue was evaluated in three consecutive trials using the semiautomatic in vitro technique for gas production described by Maurício et al. (1999), adapted from Fedorah & Hrudey (1983). Samples of 500 mg were assessed in incubation flasks with an internal volume of 50 mL for the collection of gas samples for the quantification of CH_4 (Terry et al., 2016; Oliveira et al., 2018).

The treatments consisted of the following diets: corn silage (control); and protein concentrate with soybean meal and a mineral premix, included at three levels

(25, 50, and 75%) in the dry matter (DM). All diets were incubated together with the inoculum.

Samples with the concentrate and silage (Table 1) were analyzed for: DM, at 105ºC; crude protein, by the Kjeldahl method according to Detmann et al. (2012); and neutral detergent fiber (FDN) and acid detergent fiber (FDA), as described by Van Soest et al. (1991), using the Ankom 200 fiber analyzer (Ankom Technology, Macedon, NJ, USA). Ethereal extract was determined by the extraction for 8 hours of soluble substances in petroleum ether through the Soxhlet method. Ash was obtained by combustion in a muffle furnace at 600ºC. Organic matter (OM) was calculated as the difference between the contents before and after the complete burning of the sample (OM = 100 - ash). Starch, total digestible nutrients (TDN), nonfibrous carbohydrates (NFC), and digestible energy were determined according to Compêndio Brasileiro de Alimentação Animal (2013).

The ruminal inoculum was removed and transported in a preheated thermo flask. The ruminal liquid was filtered with two layers of cotton gauzes, subjected to a continuous injection of CO_2, and kept in a thermostatic bath heated to 39°C. A buffer solution was added to the inoculum, at a ratio of 1.0 mL rumen liquid for each 6.85 mL buffer (Menke et al., 1979). After the solution was prepared, 25 mL were added to 50 mL pre-carbonated with CO_2 bottles already with 500 mg substrate diets ground in a 1.0-mm sieve. Five hours before incubation, the bottles were conditioned in a chamber at 39°C for temperature stabilization. Bottles without extracts (standard) and flasks containing only rumen liquid (white) were also included in triplicate for each inoculum and experimental period. The vials were sealed with a silicone cap and an aluminum ring and then incubated at 39°C for up to 96 hours, being shaken at 90 motions per minute. The volumes within the vials were gauged after 2, 4, 6, 8, 10, 12, 14, 17, 20, 24, 28, 34, 48, 72, and 96 hours of incubation, using a water displacement device (Fedorah & Hrudey, 1983).

At 24 hours of incubation, approximately 10 mL gas were collected from each vial with a syringe, transferred to a 6.8-mL Exetainer vial (Labco Limited, Lampeter, Ceredigion, United Kingdom), and sent to a laboratory for the quantification of CH_4 using the 7820A gas chromatography system (Agilent, Santa Clara, CA, USA) according to Holtshausen et al. (2009). After the gas production readings were completed at 24 or 96 hours, the residues resulting from incubation were collected and filtered on vacuum filter paper. These residues were oven-dried at 105ºC

Table 1. Bromatological composition of the bovine diets, containing different levels of concentrate (soybean meal and minerals) and corn silage (control), used for in vitro incubation.

Component[(1)]	Concentrate[(2)]	Silage	Inclusion of concentrate (%)		
			Silage + 25%	Silage + 50%	Silage + 75%
Dry matter (DM, %)	91.5	34.1	48.5	62.8	77.2
Organic matter (%)	89.5	95.4	93.9	92.4	90.9
Crude protein (% DM)	42	9.1	17.3	25.5	33.8
ADF (% DM)	11.5	25.6	22.1	18.5	15
NDF (% DM)	19.8	40.6	35.4	30.2	25
Ethereal extract (% DM)	1.4	3.2	2.8	2.3	1.9
NFC (% DM)	29.9	43.4	40	36.6	33.3
Starch (% MS)	8.9	32.4	26.5	20.6	14.7
TDN (%)	75.9	68.9	70.7	72.4	74.2
DE (kcal g^{-1})	3.35	3.04	3.11	3.19	3.27

[(1)]DM, dry matter; ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, nonfibrous carbohydrates; TDN, total digestible nutrients; and DE, digestible energy. [(2)]The concentrate was composed of 97% soybean meal and 3% minerals.

until reaching constant weight, and the results were used to calculate in vitro DM degradation.

After filtration, two samples of the liquid phase of each flask were collected and frozen at -20ºC for the analysis of volatile fatty acids (VFA) and ammonia nitrogen ($N\text{-}NH_3$). The $N\text{-}NH_3$ concentration was determined in a Kjeldahl micro apparatus through the distillation of 2.0 mL liquid contents recovered from the fermentation flask using 10 mL potassium chloride at 15%, 2.0 g magnesium oxide, 20 mL boric acid at 4% as the receiving solution, and hydrochloric acid (0.01 N) as the titration solution. The VFA contents were evaluated using the e2695 module for high-performance liquid chromatography (Waters Corporation, Milford, MA, USA).

Digestible energy was calculated considering 1.0 kg TDN equal to 4,409 Mcal (Silva & Leão, 1979), and energy loss was obtained as a percentage of the digestible energy of the sample, assuming the value of 9.45 kcal L^{-1} CH_4 (Brouwer, 1965).

The data were subjected to the analysis of variance considering the effects of the addition or not of the propolis residue.

Using the Gauss-Newton algorithm, the results of accumulated in vitro gas production were adjusted to the bicompartmental logistic model described by Schofield et al. (1994), according to the equation: $V(t) = Vf1 / (1 + \exp(2 - 4 \times c1 \times (T - L))) + Vf2 / (1 + \exp(2 - 4 \times c2 \times (T - L)))$, where V(t) is the maximum total volume of gases; Vf1 is the maximum volume of the gases produced by the slow degradation fraction made up of fibrous carbohydrates (FC); c1 is the rate of gas production by the degradation of FC; L is the latency phase, i.e., time of incubation; T is fermentation time; Vf2 is the maximum volume of the gases produced by the fast degradation fraction made up of NFC; and c2 is the rate of gas production by the degradation of NFC.

The data were analyzed considering the fixed effects of the food additive (use or not of the propolis extract residue), type of diet (inclusion levels of the concentrate based on soybean meal), and interaction between these treatments and the random effect of the incubation round (n = 3 rounds). Statistical significance was considered when $p \leq 0.05$. Treatments were compared regarding diet type when $p < 0.05$, considering the linear and quadratic effects associated with the 0, 25, 50, and 75% inclusion levels of the concentrate based on soybean meal. In addition, a correlation study was performed between the variables using Pearson's correlation coefficient, at 5% probability.

Results and Discussion

The production of gases (Vf1) from FC was affected by the increasing levels of the concentrate and by the inclusion of the propolis residue (Table 2). The addition of soybean meal also affected the rate of gas production (c1) due to the fermentation of FC, reducing linearly as the level of concentrate increased. The highest Vf1 occurred with the highest concentrate level associated with the presence of the residue. Since the concentration of FC decreased as soybean meal was increased in the diets, it is possible to increase the gas production from this carbohydrate, which was maximized with the use of the propolis residue.

The availability of NFC in the rumen is essential for ruminal microbial growth and a greater microbial protein intake (Cabral et al., 2011), with a consequently higher rate of fermentation and gas production. In this way, the increase of NFC and reduction of the amount of FC to be fermented may have maximized the performance of the cellulolytic and hemicellulolytic bacteria, increasing Vf1. This result differs from those of Araujo et al. (2018), who analyzed in vitro gas production with increasing levels of a propolis ethanolic extract in diets containing 50% corn and soybean concentrate and 50% elephant grass (*Pennisetum purpureum* Schumach.), finding no change in the production of gases from FC. Therefore, the ethanolic extract and the residue of propolis somehow respond differently in terms of gas production from FC. A possible explanation is that the alcohol contained in the extract and that can inhibit bacterial growth (Patterson & Ricke, 2015) is no longer present in the propolis residue.

The production of gases from the fermentation of NFC (Vf2) was not influenced by the treatments, proving that the propolis residue, as well as the propolis extract tested in the literature, does not hinder the growth of Gram-negative bacteria related to the degradation of NFC (Soltan et al., 2016).

The addition of the propolis residue did not alter the total volume of gases produced [V (t)]; however, the inclusion of the concentrate had a positive quadratic effect. The increase in V (t) is related to a greater

amount of FC and a greater availability of protein and energy, which decreased colonization time (lag time). This result is positive, since a longer colonization time would result both in a slow growth of ruminal microorganisms, reducing the use efficiency of the diet, and in a low synthesis of microbial cells (Regadas Filho et al., 2011).

DM degradation was not affected by the addition of the propolis residue, but was higher when the concentrate was added, with a linear effect. The use of soybean meal increased the proportion of digestible nutrients, observed by the increment in TDN, which may be related to the increase in DM digestibility.

The production of total VFA was not altered by the treatments. However, the acetate:propionate ratio was influenced by the addition of the propolis residue, which resulted in a greater propionate production (Table 3). Diets containing more NFC produce greater proportions of propionate than those with higher FC amounts (El-Waziry, 2007); however, in the present study, the addition of the propolis residue, even to the control group containing only corn silage but more FC, showed a superior propionate production. This latter result differs from those observed by Prado et al. (2010) when using an alcoholic propolis extract and sodium monensin, confirming the different behavior of the propolis residue.

The addition of the propolis residue did not affect pH, differently from the inclusion of the concentrate. However, the observed pH range, between 6 and 7, allows the ruminal development of all the components of the microbial biomass, including bacteria, protozoa, and fungi (Hobson & Stewart, 1997). Lindberg (1985) found a pH range from 6 to 8, compatible with the maximum action of the enzymes of ruminal microorganisms. According to Mould et al. (1983), only when the pH is below 6 do deleterious effects on FC degradation begin, which is not desirable.

Table 2. Parameters of the in vitro ruminal kinetics of bovine diets with increasing levels of concentrate based on soybean meal, with or without the use of a propolis extract residue additive[(1)].

Parameter	Additive (A)	Inclusion of concentrate (IC)				MSE	P-value			IC effect	
		0%	25%	50%	75%		A	FS	A x FS	Linear	Quadratic
Vf1 (mL)	Control	84.2	84.7	96.4	108.4	4.43	0.01	<0.001	0.33	<0.001	0.002
	Propolis residue	83	96.9	111	118.7						
c1 (mL h^{-1})	Control	0.035	0.032	0.029	0.029	0.0019	>0.50	0.01	>0.50	0.002	0.002
	Propolis residue	0.036	0.032	0.029	0.03						
Vf2 (mL)	Control	149.5	152.8	152.7	154.6	6.63	>0.50	>0.50	>0.50	-	-
	Propolis residue	147.4	153.1	151.6	144.5						
c2 (mL h^{-1})	Control	0.122	0.11	0.111	0.102	0.006	>0.50	0.21	>0.50	-	-
	Propolis residue	0.116	0.115	0.111	0.109						
V (t) (mL)	Control	233.7	237.5	249.1	263	10.61	>0.50	0.05	>0.50	0.001	0.007
	Propolis residue	230.4	250	262.6	263.2						
Lag time (hours)	Control	2.57	2.1	1.66	1.3	0.28	0.12	0.02	>0.50	0.13	0.31
	Propolis residue	2.66	2.25	2.08	1.94						
DMD (%)	Control	63.1	70	72.1	74.6	2.1	0.09	<0.001	0.42	0.02	0.06
	Propolis residue	59.1	65.3	68.2	76.3						

[(1)]Vf1, volume of the gases produced by the fermentation of fibrous carbohydrates; c1, rate of gas production by the fermentation of fibrous carbohydrates; Vf2, volume of the gases produced by the fermentation of nonfibrous carbohydrates; c2, rate of gas production by the fermentation of nonfibrous carbohydrates; V (t), total volume of the gases produced by the fermentation of the sample; Lag time, colonization time; DMD, dry matter degradation at 96 hours of incubation; A, addition or not of the propolis extract residue; MSE, mean standard error; and FS, inclusion levels of the concentrate based on soybean meal.

The concentration of N-NH_3 was not altered by the addition of the propolis residue in relation to the control, even when the protein levels of the diet were increased via concentrate. However, a linear increase in N-NH_3 was observed with the inclusion of the concentrate in the diet. These results were not expected since other studies reported an increase in the rumen nitrogen metabolism, a reduction in proteolytic bacteria, and a consequently lower concentration of free N-NH_3 with the inclusion of concentrate (Oliveira et al., 2006; Ozturk et al., 2010; Aguiar et al., 2014).

The concentrations of N-NH_3 varied from 8.4 to 7.8 mg dL^{-1} and from 17.2 to 14.4 mg dL^{-1} in the control and in the treatments with the propolis residue, respectively, from the lowest to the highest level of concentrate inclusion. The obtained ranges meet the levels required for microbial growth, which would be from 5 to 15 mg dL^{-1} N-NH_3 (Detmann et al., 2010).

In the present experiment, the addition of the propolis residue reduced the percentage of CH_4 production by 4.35, 7.00, 8.57, and 9.91% at the concentrate inclusion levels of 0, 25, 50, and 75%, respectively (Table 4). Leopoldino et al. (2007) also observed a reduction in CH_4 considering total gas production, when testing an alcoholic propolis extract as an alternative to reduce CH_4 emissions in diets with the inclusion of 46.5% concentrate to 53.5% roughage. Ehtesham et al. (2018) reported that the addition of an alcoholic extract of propolis increased the total production of gases and, simultaneously, reduced the production of CH_4 in diets with a medium or high inclusion of concentrate due to the reduction of methanogenic and protozoan bacteria, which is possibly related to the composition of the propolis used.

The propolis residue was shown to be efficient in reducing CH_4 with a greater energy retention and use of the diet by the animal, which, in practical terms, may indicate the possibility of obtaining a greater animal efficiency. Considering the addition of the propolis residue and the estimated energy loss (percentage of digestible energy), at the concentrate inclusion levels of 0, 25, 50, and 75%, the digestible energy available to the animal was of 8.11, 11.25, 10.00, and 18.52%, respectively, compared with the control.

Table 3. Concentrations and ratio of short-chain volatile fatty acids (acetate, propionate, and butyrate), pH index, and concentration of ammoniacal nitrogen at 24 hours of incubation with or without the use of propolis extract residue additive in bovine diets with increasing levels of concentrate based on soybean meal[(1)].

Volatile fatty acids	Additive (A)	Inclusion of concentrate (IC)				MSE	P-value			IC effect	
		0%	25%	50%	75%		A	FS	A x FS	Linear	Quadratic
Total VFA (mmol L^{-1})	Control	54.9	57.8	50.9	57.7	4.37	>0.50	>0.50	>0.50	-	-
	Propolis residue	55.2	63.2	53.2	62.8						
Acetate (mmol L^{-1})	Control	33.1	35.3	38.6	36.3	2.52	0.25	>0.50	>0.50	-	-
	Propolis residue	32.7	37.6	31.6	37.1						
Propionate (mmol L^{-1})	Control	11.8	12.1	11.2	12.4	0.90	0.19	>0.50	>0.50	-	-
	Propolis residue	14.2	14.6	12.6	14.5						
Butyrate (mmol L^{-1})	Control	10.0	10.4	9.0	9.0	1.33	>0.50	>0.50	>0.50	-	-
	Propolis residue	8.3	9.1	7.4	11.3						
Acetate: propionate	Control	2.8	2.9	2.8	3.0	0.15	0.01	>0.50	>0.50	-	-
	Propolis residue	2.3	2.3	2.6	2.5						
pH	Control	6.7	6.8	6.9	7.1	0.04	>0.50	<0.001	>0.50	0.09	0.21
	Propolis residue	6.8	6.9	6.9	7.0						
N-NH_3 (mg dL^{-1})	Control	8.4	11.6	13.2	17.2	0.42	0.31	0.002	>0.50	<0.001	<0.001
	Propolis residue	7.8	10.4	13.6	14.4						

[(1)]VFA, volatile fatty acids; N-NH_3, ammoniacal nitrogen; A, use or not of propolis extract residue; MSE, mean standard error; and FS, inclusion levels of the concentrate based on soybean meal.

Table 4. Production of methane (CH_4) as a percentage of gases – in vitro, as volume per unit of dry matter and, incubated, as volume per unit of digestible dry matter –, as well as energy contained in the emitted CH_4, CH_4 energy loss, and production of CH_4 as a function of the production of volatile fatty acids with or without the use of a propolis extract residue additive in bovine diets with increasing levels of a concentrate based on soybean meal[(1)].

Methane	Additive (A)	Inclusion of concentrate (IC)				MSE	P-value			IC effect	
		0%	25%	50%	75%		A	FS	A x FS	Linear	Quadratic
CH_4 (%)	Control	9.2	10.0	10.5	11.1	0.37	0.009	0.007	>0.50	0.16	0.37
	Propolis residue	8.8	9.3	9.6	10.0						
CH_4 (mL g^{-1} DM)	Control	10.9	12.1	12.5	12.8	0.43	<0.001	0.040	0.385	>0.50	>0.50
	Propolis residue	10.2	10.7	11.3	10.5						
CH_4 (mL g^{-1} DMD)	Control	26.5	29.8	29.0	28.9	2.65	0.098	>0.50	>0.50	-	-
	Propolis residue	26.2	27.6	25.8	21.4						
Loss of energy for CH_4 (% DE)	Control	7.4	8.0	8.0	8.1	0.31	0.001	0.376	>0.50	-	-
	Propolis residue	6.8	7.1	7.2	6.6						
CH_4 per total VFA (mL mol^{-1})	Control	196.6	201.3	186.3	220.8	18.04	0.15	>0.50	>0.50	-	-
	Propolis residue	182.9	187.5	136.6	266.0						

[(1)]DM, dry matter; DMD, dry matter degradation; DE, digestible energy; VFA, volatile fatty acids; A, use or not of propolis extract residue; MSE, mean standard error; and FS, inclusion levels of the concentrate based on soybean meal.

Conclusion

The inclusion of the propolis residue in bovine diets increases the production of gases from the degradation of fibrous carbohydrates, but reduces the acetate:propionate ratio and methane production.

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