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MICROBIAL PROTEIN SYNTHESIS AND NITROGEN BALANCE IN CROSSBRED HEIFERS FED WITH TWO TANNINS SOURCES

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ABSTRACT - Low to moderate concentrations of condensed tannins in ruminant diets are considered to increase the post-ruminal flow of non-ammonia nitrogen. The objective of this research was study the microbial protein synthesis and nitrogen balance of lactating cows fed with a diet of two tannin sources, based on sorghum (condensed tannin) with increasing levels of tannic acid (hydrolysable tannin). So, increasing levels of tannic acid in a sorghum-based diet for five Holstein/Zebu crossbred lactating heifers were subject to a 5 × 5 Latin square experimental design, five heifers x five tannins acid levels x five periods. The excretion of total purine derivatives, through the collection of spot urine samples, and the N concentration values in the milk and urine were used. All heifers received 9.87 kg/DM of corn silage as roughage and 6.38 kg of concentrate (not fed *ad libitum*). Diet 1 (control) contained low-tannin sorghum (0.027 kg total tannin), and the other diets contained high-tannin sorghum. The levels of tannic acid added used were established based on the quantity of condensed tannin in high-tannin sorghum. Thus, diets 2, 3, 4, and 5 were supplemented with 1.5g (13% DM), 79.5g (2.6% DM), 157.5 g (3.9% DM), and 235.5 g (5.2% DM) of tannic acid totalling 0.078, 0.156, 0.234, and 0.321 total tannin kg/day respectively. The urinary excretions of urea (2.0055), total allantoin (185.12 mmol/day^{-1}), and total purine derivatives (222.32 mmol/day^{-1}); absorbed purines 179.3 mmol/day^{-1}); the N microbian synthesis (130.358 (g)N/day^{-1}); and the concentrations of allantoin in milk (36.40 mmol/day^{-1}) were not affected by the diets. Diets using two tannins (hydrolysable and condensed) sources supplementation didn't affect the animals' health, neither improved the microbial synthesis efficiency nor the N balance for milk production.
Keywords: Creatinine, lactation, polyphenols, urea.

SÍNTESE DE PROTEÍNA MICROBIANA E BALANÇO DE NITROGÊNIO EM NOVILHAS MESTIÇAS ALIMENTADAS COM DUAS FONTES DE TANINOS

RESUMO - Concentrações baixas a moderadas de taninos condensados em dietas de ruminantes tem sido considerada por aumentando o fluxo pós-ruminal de nitrogênio não amoniacal. O objetivo desta pesquisa foi estudar a síntese proteica microbiana e o balanço de nitrogênio de vacas em lactação alimentadas com uma dieta composta de duas fontes de tanino, à base de sorgo (tanino condensado) com níveis crescentes de ácido tânico (tanino hidrolisável). Níveis crescentes de ácido tânico foram utilizados em uma dieta à base de sorgo para cinco novilhas lactantes mestiças Holandês/Zebu em um delineamento experimental quadrado latino 5 × 5. Utilizou-se a excreção de derivados totais de purina, através da coleta de amostras *spot* de urina, e os valores de concentração de N no leite e urina. Todas as novilhas receberam 9,87 kg/MS de silagem de milho como volumoso e 6,38 kg de concentrado (não alimentados *ad libitum*). A dieta 1 (controle) continha sorgo com baixo teor de tanino (0,027 kg de tanino total), e as demais dietas continham sorgo com alto teor de tanino. Os níveis de adição de ácido tânico utilizados foram estabelecidos com base na quantidade de tanino condensado no sorgo alto teor de tanino. As dietas 2, 3, 4 e 5 foram suplementadas com 1,5g (13% MS), 79,5 g (2,6% MS), 157,5 g (3,9% MS) e 235,5 g (5,2% MS) de ácido tânico. totalizando 0,078, 0,156, 0,234 e 0,321 kg de tanino total/dia respectivamente. As excreções urinárias de ureia (2,0055), alantoína total (185,12 mmol/dia^{-1}) e derivados totais de purina (222,32 mmol/dia^{-1}); purinas absorvidas 179,3 mmol/dia^{-1}), a síntese microbiana de N (130,358 (g)N/dia^{-1}) e as concentrações de alantoína no leite (36,40 mmol/dia^{-1}) não foram afetadas pelas dietas. Dietas com suplementação de duas fontes de taninos (hidrolisável e condensado) não afetaram a saúde do animal, nem melhoraram a eficiência da síntese microbiana nem o balanço de N para produção de leite.
Palavras-chave: Creatinina, lactação, polifenóis, ureia.

INTRODUCTION

Livestock supports to food security by supplying essential macro and micro-nutrients, providing manure and draught power, and many other products, which generate income to the farmer. But they also consume food edible by humans and graze on pastures that could be used for crop production (OGOT et al., 2018). In animal production systems, the feed, especially high-quality protein, where protein is one of the most expensive dietary components in ruminant nutrition (AHNERT et al., 2015) of the total

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operating costs of the production system. In addition, high-production dairy cows must be fed a sufficient amount of energy and protein. The increase in the flow of ruminal protein depends on the decrease in proteolysis by ruminal microorganisms and the increase in efficiency of microbial synthesis (HARTINGER et al., 2018).

In recent years, several reviews have evaluated the potential of plant extracts as growth promoters and natural alternatives (SANTOS et al., 2021; ZHANG et al., 2019). Polyphenols such as tannins have attracted the attention of ruminant nutritionists to form complexes with dietary proteins and protect them from microbial degradation (ZHANG et al., 2019; PATRA and SAXENA, 2011). Tannins are compounds that can improve nitrogen utilization and feed conversion efficiency, change milk fatty acid profiles, and reduce methane emission in ruminants (SANTOS et al., 2021; ZHANG et al., 2019).

Low to moderate concentrations (2–4%) of condensed tannins in ruminant diets are considered to increase the post-ruminal flow of non-ammonia nitrogen (N) due to their ability to form reversible complexes with feed proteins and thereby protect them from rumen degradation and enhance animals' protein supply (SANTOS et al., 2021).

In this context, the concept of protein nutrition in ruminants has evolved considerably in recent decades, which enabled the development of the factorial method for determining nutrient requirements used in the main protein systems available (SANTOS et al., 2011). The allantoin urinary excretion and other purine derivatives seem to be affected by changes in the endogenous microbial components proportion or by the increase in N recycling (PUCHALA and KULASEN, 1992). Therefore, it was expected that an increased tannin level would lead to an increase in the allantoin or purine derivatives amount.

N balance, under controlled conditions, provides an estimate of the protein metabolism and constitutes, corresponds to the difference between the amount of nitrogen ingested and the value excreted by urine and feces. An evaluation method of the foodstuff and the nutritional status of the animal (GERON et al., 2015). There are several routes through which tannins can improve the health and well-being of ruminants, namely by improving nutrition, preventing swelling/fermentation, preventing fly infestation in sheep, and reducing the number of gastrointestinal worms (ALONSO-DÍAZ et al., 2010), as well as livestock pollution impact. The main benefit may be related to the digestion of proteins (NAUMANN et al., 2017).

However, studies on the effects of plant-derived tannins in ruminant nutrition are conflicting. Authors found that a low concentration (7.5%) of tamarind seed husk tannins improved the daily weight gain and milk protein content of crossbred lactating cows. Dschaak et al. (2011) reported that supplementing 3% quebracho condensed tannins decreased dry matter intake (DMI), but did not affect milk production, milk composition, or N utilization efficiency for milk production. Broderick et al. (2017) showed that feeding condensed tannins reduced the concentrations of true protein and urea nitrogen in milk, and depressed the apparent nutrient digestibility. Such discrepancies can be attributed to the tannin source, type, and dietary inclusion level (ZHANG et al., 2019).

Based on their structure and reactivity, and the tannins category (hydrolyzable or condensed), some studies show no difference among tannin sources on ruminal protein degradation, total tract digestibility of dietary proteins, or animal performance (ZHANG et al., 2019). The present research aimed to study the microbial protein synthesis and nitrogen balance of lactating cows fed with a diet of two tannin sources, based on sorghum (condensed tannin) with increasing levels of tannic acid (hydrolysable tannin).

MATERIALS AND METHODS

All practices performed in the present study involving the use of animals were approved by the Institutional Animal Care of the Ethics Commission in Animal Use of the Biotechnological Centre of Federal University of Paraiba (CEUA/BIOTEC/UFPB) (protocol number 072/2016). This experiment was conducted at the Cattle Farming Unit of the Animal Science Department of the Agricultural Science Centre/Federal University of Paraiba, *Campus* II, in the municipality of Areia (Paraíba) from February to May of 2015.

Five crossbred Holstein × Zebu heifers were used, the heifers were approximately four years old (averaging 420 ± 30 kg initial (body weight) BW, 435± 32,5 kg average BW) and approximately 100 days of lactation, with an average initial production of 18 ± 4 kg/day were used in a 5 × 5 Latin square design. Each experimental period lasted 20 d, with 15 d for diet adaptation and the last 5 d for sample collection, for a total of 100 experimental days. heifers were weighed at the beginning and the end of each experimental period. The heifers were housed in individual 18 m^2 stalls with concrete floors and equipped with individual stall feeders and water dispensers. Before starting the experiment, the heifers were dewormed and treated for ectoparasites with 3.5% Ivermectin. The adaptation period to the facilities, the experimental diets, and the stabling totalled 10 d.

The experimental diet consisted of roughage and concentrate, at fixed roughage: concentrate ratio of 64:36, as a TMR (total mixed ration). The diet was offered in equal amounts twice a day at 06.00 am and 01.30 pm after milking. To ensure adequate access to the roughage, the forage intake was determined during the adaptation days, and roughage was fed at 140% of the average intake for the previous 5 days. The corn silage was produced at the Agricultural Science Centre/Federal University of Paraíba, Areia/PB.

The experimental diet was formulated to meet the lactating demands according to recommendations from the National Research Council (NRC) (2001). All heifers received 9.87 kg/DM (dry matter) of corn silage as roughage and 6.38 kg of concentrate consisting of 2.58 kg/DM of ground sorghum, 0.87 kg/DM of cornmeal, 1.32 kg/DM of soybean bran, 0.44 kg/DM of wheat bran, 0.2 kg/ DM of urea and 0.18 kg/ DM of the mineral mixture

(Bovigold® DSM) daily. The nutritional ingredients compositions of the dietary are outlined in Table 1.

Two sorghum cultivars were used to prepare the diet, diet 1 (control diet) contained BRS Ponta Negra (low tannin) cultivar sorghum (0.92% total condensed tannin in the DM) to provide the lowest possible number of tannins. Diets 2, 3, and 4 contained A9904 (hight tannin) cultivar sorghum (2.55% total condensed tannin in the DM), to provide the highest quantity of available tannins possible, in order to guarantee the tannin effects in animal metabolism. The tannic acid levels added to the diets were established based on the analysis of the condensed tannin quantity in high-tannin sorghum. The levels of total condensed tannin (DM) were calculated according to the HCl-butanol method (NAUMANN et al., 2017) and the total tannin in the DM according to Terril et al. (1992) method.

TABLE 1 - Chemical composition, dietary ingredients proportion, condensed tannin, and hydrolysable tannin (tannic acid) concentration in experimental diets in kilogram (kg) of natural (corn silage) matter. And nutritional composition of diets ingredients on a dry matter (%) basis.

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	DM	CP	EE	NDF	MM	NFC	TCH
	---------- kg ----------					---------- % DM ----------						
Corn silage	35.00	35.00	35.00	35.00	35.00	28.2	8.0	2.2	55.1	4.8	33.6	84.8
Ground corn	1.00	1.00	1.00	1.00	1.00	86.7	9.0	4.3	14.0	1.2	74.5	80.6
Soybean meal	1.50	1.50	1.50	1.50	1.50	88.3	48.8	2.0	14.5	6.1	30.4	43.5
Wheat meal)	0.50	0.50	0.50	0.50	0.50	87.7	16.4	1.7	44.5	6.3	30.1	74.0
Urea	0.20	0.20	0.20	0.20	0.20	100	283	0.0	0.0	0.0	0.0	0.0
Sorghum A9904	0.00	3.00	3.00	3.00	3.00	85.2	10.9	2.7	14.0	3.3	73.9	82.4
Sorghum control	3.00	0.00	0.00	0.00	0.00	87.5	9.3	2.9	13.8	3.5	73.9	84.4
Mineral mix *	0.18	0.18	0.18	0.18	0.18	100	-	-	-	100	-	-
Tannic acid	0.00	0.0015	0.0795	0.158	0.236	100	-	-	-	-	-	-
Total	41.38	41.40	41.46	41.54	41.62							

Dry matter (DM), crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), mineral matter (MM), non-fibrous carbohydrate (NFC), total carbohydrate (TCH). *190 g kg^{-1} Ca, 60 g kg^{-1} P, 20 g kg^{-1} S, 20 g kg^{-1} Mg, 35 g kg^{-1} K, 70 g kg^{-1} Na, 15 mg kg^{-1} Co, 700 mg kg^{-1} Cu, 10 mg kg^{-1} Cr, 700 mg kg^{-1} Fe, 40 mg kg^{-1} I, 1600 mg kg^{-1} Mn, 19 mg kg^{-1} Se, 2500 mg kg^{-1} Zn, 400000 IU kg^{-1} vitamin A, 100000 IU kg^{-1} vitamin D3, 2400 IU kg^{-1} vitamin E.

Tannic acid ($C_{76}H_{52}O_{46}$) P.A. purchased from Anidrol® laboratory products, São Paulo - Brazil, a purified powder product was added to prepare the increasing tannin doses. Predetermined proportions of powder tannic acid (based on the quantity of condensed tannin from sorghum A9904, 1.27, we've added, continuously, this same quantity, therefore, 2.6, 3.9, and 5.2, to find the final total percentages) were mixed with the concentrate on the day before trough feeding. Thus, diets 2, 3, 4, and 5 were supplemented with 1.5, 79.5, 157.5, and 235.5 g of tannic acid respectively, and provided an increase in the dietary total tannin percentages as outlined in Table 2.

Therefore, the following tannin sources were provided in the experimental diets: condensed tannins through both sorghum cultivars and hydrolyzable tannins through tannic acid. Water was provided to the animal's *ad libitum*. Experimental diets nutritional composition (concentrate plus roughage) (%DM) were, diet 1 (control diet) 36.85 DM, 15.55 CP, 1.99 EE, 38.66 NDF, 5.48 MM, 33.68 NFC and total tannin 0,46% and to Diets 2, 3, 4 and 5, 37.30 DM, 15.46 CP, 2.08 EE, 38.66 NDF, 5.67 MM 33.24 NFC and total tannin 1.30%, 2.60%, 3.90% and 5.2% respectively.

Fecal samples output was collected immediately after each spontaneous defecation from day 15 to 20 of each period, weighed, stored in aluminum plates, and oven-dried (55°C) (immediately after each collection day) for 72 h, and then weighed and ground in a Wiley mill (model 3; Arthur H. Thomas, Philadelphia, PA) to pass through a 2-mm screen and blended manually at the end of each period to obtain pooled samples per treatment in proportion to the daily excretion to measure digestibility. After grinding, half of each ground sample was ground again to pass through a 1-mm screen, and stored in a freezer, -20ºC, for later analysis.

Milk production was recorded daily by individual weighing (kg/day). Milk samples were collected twice daily (morning and afternoon) from day 15 to 20 of each period. Milk samples from the morning milking were stored in a plastic container and refrigerated, with no preservatives, for subsequent mixing with milk samples from the afternoon milking to form a composite sample/ heifers /day. Sterile plastic bottles with 100- and 200-mL capacities were used for the collections and then stored in a freezer at -20ºC for subsequent analysis.

TABLE 2 - Percent and kg contributions of condensed tannin in sorghum and supplemented tannic acid in the experimental diets.

Diets	Contribution of sorghum CT (%)	Addition of tannic acid. HT (%)	Contribution of sorghum CT (3 kg)	Addition of tannic acid (HT) (kg/day)	Total tannin in diet (CT + HT) (%)	Total tannin (CT + HT) (kg/day)
Diet 1	0.46	0.00	0.0276	0.0000	0.46	0.0276
Diet 2	1.27	0.03	0.0765	0.0150	1.30	0.0915
Diet 3	1.27	1.33	0.0765	0.0795	2.60	0.1560
Diet 4	1.27	2.63	0.0765	0.1575	3.90	0.2340
Diet 5	1.27	3.93	0.0765	0.2355	5.20	0.3210

Condensed tannin (CT), hydrolysable tannin (HT).

From day 15 to 20 of each period (day 1, 3, and 5) were collected in the morning period, 4 h after feeding the animals by jugular venipuncture. The blood was collected into 10-mL vacuolated tubes. Immediately after collection, the samples were cooled prior to the performance of a complete blood count (without clot activators). Plasma was separated from another portion of the collected blood (with clot activators) through centrifugation at 3000 rpm for 15 min. within 45 minutes of collection. Plasma samples were stored in a freezer -20ºC for later analysis.

Urine spot samples were collected from d 15 to 20 of each period. Urine spot samples were obtained from all animals, in the morning, 4 h after feeding, by spontaneous or massage-stimulated urination. From the collected urine, after homogenization and filtration (with gauze), two samples were obtained per animal, where one consisted of pure urine and the other of an aliquot of 10 mL of urine that was diluted in 40 mL of 0.036 N sulfuric acid to prevent the bacterial degradation of purine derivatives and the precipitation of uric acid, as described by Valadares Filho et al. (1999), for the allantoin analysis. Then, the samples were placed in plastic containers and stored in a freezer at -20ºC for later analysis. The estimated urinary volume (L) was obtained by the Equation 1, according to Chizzotti et al. (2008). The mean of 24.04 (mg/kg BW) was obtained from the studies by Chizzotti et al. (2008) to obtain the total daily excretion of creatinine.

$$[(24.04 \text{ x bw mean})/\text{creatinine value}/10] \quad \text{(Equation 1)}$$

Where:
bw = body weigth.

Pooled samples of each material ground through 1-mm sieves (roughage, feces, orts, and diets) were analyzed according to the standard analytical procedures of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA; DETMANN et al., 2012) for DM (dried overnight at 105°C; method INCT-CA number G-003/1), ash (complete combustion in a muffle furnace at 600°C for 4 h; method INCT-CA number M-001/1). The crude protein (CP) and total nitrogen (N) levels (Kjeldahl procedure; method INCT-CA number N-001/1) and a correction factor of 6.25, ether extract was conducted in a reflux system (Soxtherm, Gerhardt, Germany) by Randall procedure; method INCT-CA number G-005/1. The NFC levels were calculated using the equation proposed by Hall et al. (2000) and digestibility test.

The experimental diets were analyzed in the Animal Nutrition Laboratory of the CCA/DZ/UFCP to determine the DM, MM, EE, and CP contents according to Detmann et al. (2012). The assessment of NDF was performed according to the methods by Detmann et al. (2012), and gross energy was assessed by sample oxidation in a bomb calorimeter. The total digestible nutrient (TDN) values were calculated according to the NRC (2001). The total carbohydrates (TCH) were calculated according to Sniffen et al. (1992) (Equation 2). The assessment of the neutral detergent fiber (NDF), neutral detergent insoluble nitrogen (NDIN) and neutral detergent insoluble protein (NDIP) levels was performed according to INCT - CA F-002/1, by Detmann et al. (2012) (Equations 3, 4 and 5).

$$\text{TCH} = 100 - (\%\text{CP} + \%\text{EE} + \%\text{Ash}) \quad \text{(Equation 2)}$$

$$\text{NFC \% TCH \% NDFcp, with NDF corrected for ash and protein} \quad \text{(Equation 3)}$$

$$\text{TDNI} = \left(\text{DCPI} + \text{DNFCI} + \text{NDFI} + (\text{DEEI X } 2.25)\right) \quad \text{(Equation 4)}$$

$$\% \text{ TDN} = (\text{TDNI}/\text{DMI})\text{X } 100 \quad \text{(Equation 5)}$$

Where:
TDNI = total digestible nutrient intake,
DCPI = digestible CP (crude protein) intake,
DNFCI = digestible non-fibrous carbohydrate intake,
NDFI = NDF (neutral detergent fiber) intake,
DEEI = digestible EE (ether extract) intake and
DMI = DM (dry matter) intake.

The analyses of the urea, uric acid and creatinine of the urine collected and frozen in its pure state were analyzed, using a LABMAX 240 automated biochemistry analyzer (2231.110.111), in the Laboratory of the Veterinary Hospital of UFRPE. The allantoin analysis in the acidified urine were analyzed according to the method of Chen and Gomes (1992). Total urinary N was obtained by the Kjeldahl procedure (method INCT-CA number N-001/1). The 4% fat-corrected milk production (FCMP) was calculated using the formula of Gaines (1928) proposed by the NRC (2001) as the Equation 6.

$$\text{FCMP (4\%)} = [(\text{MP} \times 0.4)\ (\%\ \text{Fmilk} \times 0.15)] \quad \text{(Equation 6)}$$

Where:
FCMP = fat-corrected milk production,
MP = kg milk produced and
%Fmilk = milk fat percentage

The milk total nitrogen levels were assessed using the method of Kjeldahl (2000) with the correction factor adapted for milk of 6.38 (Randall procedure; method INCT-CA number G-005/1). For the fat determination, the Folch method was used (FOLCH et al., 1957). The analysis of milk allantoin was performed in the Milk Laboratory of the Cattle Farming Unit of the Animal Science Department of the Agricultural Science Centre of UFRPE according to Chen and Gomes (1992).

The estimated urinary volume (L) was according to (CHIZZOTTI et al., 2008). The total excretion of purine derivatives was calculated by the sum of the amounts of allantoin and uric acid excreted in the urine and the amount of allantoin excreted in the milk, expressed in mmol/day. The absorbed purines (X, mmol/day) were calculated from the excretion of purine derivatives (Y, mmol/day) by the Equation 7.

$$Y = 0.85x + 0.385\ P^{0.75} \quad \text{(Equation 7)}$$

Where:
Y = excretion of purine derivatives,
0.85 = the recovery of purines absorbed as purine derivatives,
X = absorbed purines
$0.385 \times P^{0.75}$ = endogenous contribution to purine excretion and
P0.75 = metabolic weight (VERBIC et al., 1990).

The synthesis of microbial nitrogen compounds in the rumen (Y, g N/day) was calculated as a function of the absorbed purines (X, mmol/day) by the Equation 8.

$$Y = (70x) / (0.83 \text{ x } 0.116 \text{ x } 1000) \quad \text{(Equation 8)}$$

Where:
70 = N content in the purines (mg N $mmol^{-1}$),
0.83 = digestibility of microbial purines and
0.116 = ratio N-purine: total N in the bacterias (CHEN and GOMES, 1992).

The efficiency of dietary nitrogen use was calculated according to the ratio between the nitrogen excreted in the milk as crude protein and the nitrogen consumed. The apparent nitrogen balance was calculated by the following Equations 9, 10 and 11, expressed in g/day and in $g/kg^{0.75}$/day.

$$N_{retained} = N_{ingested} - (N_{feces} + N_{urine} + N_{milk}) \quad \text{(Equation 9)}$$

$$N_{absorbed} = N_{ingested} - N_{feces} \quad \text{(Equation 10)}$$

$$N_{ingested} = N_{offered} - N_{leftovers} \quad \text{(Equation 11)}$$

Statistical analyses were performed using the MIXED procedure of SAS 9.4 (SAS 2010, Inst. Inc., Cary, NC) according to a 5 × 5 Latin square design including the fixed effect of treatment and the random effects of heifers and experimental period. Statistical significance was considered at $p \leq 0.05$. The Equation 12 of the statistical model was:

$$yijk = m + li + cj + tk(ij) + eijk \quad \text{(Equation 12)}$$

Where:
yijk = value observed at the experimental unit which received the k treatment (at line i and column j),
m = effect of the general mean,
li = line i effect,
cj = column j effect,
tk(ij) = k treatment effect applied at line i and column j and
eijk = random error (residue).

At the analysis, the coefficients were analysed by the minimum square method. It was applied the Normality test (Kolmogorov). The Delta was always the same.

RESULTS AND DISCUSSION

The milk yield (kg/day) 15.7, 16.0, 15.8, 15.4, and 14.7 had a linear effect ($p>0.05$), with a reduction of 249 g for each percentage unit of tannin in the diet, with regression equation, Y = -0.249x + 16.271, R^2 = 67%, decreasing on average 0.350 kg of milk as the tannin inclusion was increased. The milk production corrected for 4% fat (14.1, 14.0, 13.3, 14.7 and 14.2 kg/ heifers/day) and the dry matter intake (14.8, 14.4, 14.9, 14.8 and 14.1 kg/heifers/day) did not differ ($p>0.05$) significantly between diets. The estimated urinary volume in L/heifers/day was influenced ($p>0.05$) by tannin levels, with a mean of 15.42 L/ heifers /day (Table 3). The estimated urinary volume was 15.43 L/heifers /day and had a linear effect ($p>0.05$), with regression equation, Y = -1.387x + 19.585, R^2 = 76%, decreasing as the tannin inclusion was increased.

TABLE 3 - Urinary volume, uric acid, urea, creatinine, urinary nitrogen, urinary nitrogen/ creatinine ratio, urine allantoin, milk allantoin, total allantoin, total and absorbed purines, microbial nitrogen synthesis and microbial crude protein of cows fed with sorghum and different tannic acid levels.

Variable	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	SEM	P-value	
							L	Q
Estimated urinary volume (L)*	19.4	15.5	15.6	12.7	13.9	3.62	0.01*	0.26
Uric acid (mmol/day)	32.9	37.4	35.7	42.2	37.8	7.40	0.18	0.45
Urea (mg/kgPV)	2.01	1.98	1.97	2.18	1.89	375.81	0.93	0.63
Creatinine (mmol/day)**	59.2	71.7	68.1	83.0	78.7	12.86	0.01*	0.49
Un (mg/dL)	6.8	7.7	6.7	7.9	7.7	1.60	0.39	0.98
Un/Uc	0.12	0.11	0.10	0.10	0.10	-	-	-
Urine allantoin (mmol/day)	151.9	143.5	136.8	154.9	156.1	44.31	0.75	0.85
Milk allantoin (mmol/day)	38.0	33.5	40.3	40.9	29.2	8.19	0.40	0.16
Total allantoin (mmol/day)	190.0	176.9	177.1	195.9	185.6	41.73	0.86	0.61
Total purine derivatives (mmol/day)	222.9	214.3	212.8	238.1	223.5	40.76	0.67	0.54
Absorbed purines (mmol/day)	179.8	171.3	169.7	195.5	180.2	40.45	0.67	0.54
N microbian synthesis (g N/day)	130.7	124.5	123.4	142.1	131.0	29.41	0.67	0.54
Mic CP (g N/day)	817.2	778.3	771.0	888.2	819.0	183.79	0.67	0.79

Estimated urinary volume (L)-[(24.05x bw mean)/creatinine value/10]. *Differed statistically according to analysis of variance and regression analysis at a 5% level of significance, **Creatinine: Y = 5.04x + 57.04, R^2 = 73%. urinary nitrogen (Un), urinary creatinine (Uc), Un/Uc ratio, microbial nitrogen synthesis (N microbial synthesis), microbial crude protein (Mic CP). Standard error mean (SEM), probability (P), quadratic (Q), linear (L).

Urinary excretions of urea, allantoin, purine derivatives, and absorbed purines were not influenced by the addition of tannin to diets. The mean creatinine excretion was 72.16 mmol/day and increased linearly (*p>0.05*) between the diets evaluated (Table 4), with a minimum value of 59.23 mmol/day for diet 1 and a maximum value of 83.05 mmol/day for diet 4.

The mean uric acid excretion was 37.21 mmol/day and did not differ (*p>0.05*) between treatments. The mean excretion of total allantoin was 185.12 mmol/day, of milk allantoin was 36.4 mmol/day and of urinary allantoin was 148.72 mmol/day, with no difference (*p>0.05*) between treatments.

The urinary urea did not differ (*p>0.05*) between treatments. The mean urea excretion in this experiment was 2004.4 mmol/day. Microbial nitrogen synthesis and microbial crude protein means were 130.36 and 814.74 respectively. The total purines derivatives and absorbed purines means were 222.32 and 179.3, respectively. With no difference (*p>0.05*) between treatments.

As can be observed in Table 4, the N balance was positive and did not differ (*p>0.05*) between treatments. The urinary excretion of N found in this experiment was 177.96 g/day, whereas it was 62.8 g/day for the excretion of fecal N and 79.8 g/day for the excretion of milk N; the mean intake was 367.65 g/day. Sorghum is a widely produced cereal in the world, it's an excellent energy source for animal nutrition. It is found in its composition, phenolic compounds such as tannin, which is the most profuse compound in plants after cellulose, hemicellulose, and lignin, it is soluble in water, acetone, and alcohol. In plants, they may be found as condensed tannins or hydrolysable tannins. The simplest form of hydrolysable tannin, the tannic acid, can be degraded by ruminal microorganisms.

TABLE 4 - Nitrogen (N) balance (g/day) of cows fed with sorghum and different tannic acid levels.

Variables	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	SEM	P-value	
							L	Q
N_{intake} (g/day)	388.0	380.8	360.0	372.6	382.0	16.02	0.79	0.21
N_{feces} (g)	67.7	61.0	63.1	67.8	54.3	16.58	0.16	0.30
N_{urine} (g)	215.8	209.3	131.7	165.0	168.0	33.22	0.07	0.27
N_{milk} (g)	72.8	83.1	68.1	97.6	77.4	9.18	0.17	0.36
$N_{excreted\ total}$ (g)	140.7	145.9	155.3	155.4	142.8	27.05	0.39	0.93
$N_{balance}$	247.2	242.4	232.4	180.6	242.9	22.80	0.50	0.61

N = nitrogen, P = probability, Q = quadratic, L = linear, SEM = standard error mean. The apparent nitrogen balance was calculated by the following equations, and expressed in g/day and in $g/kg^{0.75}$/day: BN or $N_{retained} = N_{intake} - (N_{feces} + N_{urine} + N_{milk})$; $N_{absorbed} = N_{intake} - N_{feces}$ and $N_{intake} = N_{offered} - N_{leftovers}$.

In ruminants, diets with tannins may reduce ruminal degradation of food protein, which leads to the increase of essential amino acids flow and non-ammonia nitrogen to the small intestine, and higher efficiency of microbial protein synthesis. As a result, there is less excretion of N by the feces and urine, and an increase in the efficiency of N balance.

The dry matter intake of 14.75 to 14.08 kg/heifers/day did not differ. Zhang et al. (2019) investigated the effect of tannin sources (condensed and

hydrolysable tannins) on nutrient intake, digestibility, performance, nitrogen utilization, and blood parameters in lactating dairy heifers. And, also don't observe a significant effect on DM, intake (19.39 to 20.92 kg/heifers/day). In this experiment, the tannin intake (condensed and tannic acid) ranged from 27 g/day to 321 g/day without affecting the intake significantly.

Tannins are usually associated with decreased palatability and, consequently, with an influence on the time spent eating, factors not observed in this experiment, possibly because the tannin concentration offered was not excessive. The literature shows that moderate amounts of some types of tannins ingested by ruminants have the potential to improve productivity and dietary protein utilization efficiency in the small intestine, to reduce the negative impact of gastrointestinal parasites, and to reduce urea excretion via urine and methane emission into the environment (SANTOS et al., 2021).

The estimated urinary volume, with a mean of 15.43 L/heifer/day, was influenced by tannin levels. The N balance did not differ between treatments. Zhang et al. (2019), also observed that tannin supplements did not affect the nitrogen utilization efficiency, but BCT diet (3% bayberry condensed tannins) decreased the nitrogen excretion and increased the nitrogen retention in lactating dairy heifers. Consistent previous studies already observed that tannin supplements altered nitrogen excretion (BRODERICK, 2017).

The mean creatinine excretion was 72.16 mmol/day and the values increased linearly among the evaluated diets, with a minimum value of 59.23 mmol/day and a maximum value of 83.05 mmol/day. Barbosa et al. (2006) assessed the effect of the urine collection period on the urinary excretion of creatinine in Nellore animals of four different categories (heifers, steers, bulls, and lactating heifers). The authors found mean values of urinary creatinine of 0.95 mmol/kg$^{0.75}$ for heifers and 1.18 mmol/kg$^{0.75}$ for lactating heifers. The result obtained in this experiment was 0.75 mmol/kg$^{0.75}$, i.e. lower values, demonstrating that the addition of tannins to the diet did not result in increases in creatinine levels nor renal overload. The negative tannin effect is dose-dependent. Therefore, small tannin quantities may benefit ruminants by protecting proteins from ruminal bacterial degradation and preventing fermentation (NAUMANN et al., 2017).

Creatinine is a product of the decarboxylation of phosphocreatine, which is used in skeletal muscle contraction. Its excretion is only performed renally, and creatinine is the best marker of kidney function because when there is a reduction in the glomerular filtrate, the creatinine levels in the serum increase, which indicates renal function impairment. However, creatinine alone cannot be used as an indicator of proteinuria or excessive renal load, and a determination of the Up/Uc ratio is needed, which, in random urine samples, has a predictive value for 24-h protein excretion (BOTELHO et al., 2012). Allantoin and uric acid are recent ruminal metabolism indicators, indirectly informing the number of microorganisms present in the rumen, which increase in number based on the nutritional quality and food ingestion by the ruminant (PUCHALA and KULASEK, 1992).

The urinary urea also did not differ between treatments. Blood urea nitrogen can also be used to monitor nitrogen metabolism in ruminants, and higher blood urea nitrogen can indicate higher ruminal protein degradation (ZHANG et al., 2019). The measurement of both creatinine and urea provides a better indication of the probable renal dysfunctions. The mean urea excretion found in this experiment was 712.3 mmol/day. Tannins decrease the rate of protein degradation in the rumen, thus reducing the N availability for ruminal microorganisms (PATRA and SAXENA, 2011). However, this did not occur in this experiment, possibly due to the type of tannin used in the experimental diets.

As observed in Table 4, the N balance was positive and did not differ between treatments. The N balance is very important because it can be used to quantify animal protein metabolism and analyse if there are protein gains or losses, therefore preventing productive, reproductive, environmental, and economic losses. Dschaak et al. (2011) related that supplementation of quebracho condensed tannin extract in lactating dairy cow's diets changed the N excretion route, led to less urinary N excretion but more fecal N excretion, and did not affect the N utilization efficiency for milk production. In this experiment only the estimated urinary volume was affected, decreasing as the tannin inclusion was increased. Shifting the nitrogen excretion route from the urine to the feces and forming tannin-protein complexes benefit the environment.

Firstly, according to Patra and Saxena (2011), fecal nitrogen is primarily in the organic form, which is less volatile, whereas urinary nitrogen is largely in the form of urea, which is rapidly hydrolysed to ammonia and nitrified to nitrate. Nitrate can leach into groundwater, causing water pollution, and can be converted to nitrous oxide (a greenhouse gas), accounting for approximately 65% of global anthropogenic nitrous oxide emissions. Secondly, tannin-protein complexes in the feces dissociate slowly in the soil, because mineralization of the complex is inhibited, and these feces decompose more slowly than do feces without CTs. Therefore, decreased urinary nitrogen excretion may possibly reduce ammonia and nitrous oxide emissions into the atmosphere. Excreted nitrogen via feces is more environmentally friendly than that excreted via urine (ZHANG et al., 2019).

Cows lose water primarily through milk production, in addition to fecal and urinary routes. Losses by milk and feces are similar and correspond to approximately 35% of the total water intake, whereas the urinary losses vary from 15 to 21% in lactating cows (NCR, 2001). The water requirement by cows, according to the NRC, is on average 84 kg/day, which leads to mean urinary production of 14.3 ± 3.6 L/day, which is within the values found in this experiment.

Some studies showed no difference among tannin sources on ruminal protein degradation, total tract digestibility of dietary proteins, or animal performance. Liu et al. (2011) found no toxicity to sheep supplied with 3%

HTs. Most previous studies used only one type of tannin, and few studies have compared the effects of HTs and CTs on production performance in lactating dairy cows. Therefore, this study found no effects of different tannin sources on nutrient intake, digestibility, performance, nitrogen utilization, and blood parameters in lactating dairy cows.

CONCLUSION

Diets using two tannins (hydrolysable and condensed) sources supplementation didn't affect the animals' health, neither improved the microbial synthesis efficiency nor the N balance for milk production.

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