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Effects of tannins and Monensin in a feedlot diet on in vitro ruminal fermentation[1]

Efeitos de taninos e monensina em uma ração para ovinos em confinamento sobre os parâmetros da fermentação ruminal *in vitro*

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Abstract

The objective of this study was to evaluate the effects of tannins versus Monensin on *in vitro* ruminal fermentation of a feedlot diet. The treatments were: control (no additives); low tannin (2 mg g DM^{-1}); medium tannin (4 mg g DM^{-1}), high tannin (6 mg g DM^{-1}), and Monensin (0.02 mg g DM^{-1}). The substrate was a feedlot diet composed by hay and concentrate (15:85 w/w; DM basis). Ruminal fluid was obtained from three rumen-cannulated male Santa Inês sheep. *In vitro* incubations were carried out during four consecutive weeks (run). Gas production (GP) was recorded at 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, 24, 30, 36, 42, 48, 60, 72, 84, and 96 h of incubation. At 48 and 96 h, two bottles per treatment were withdrawn to measure pH, ammonia concentration (NH_3), volatile fatty acid (VFA), *in vitro* dry matter digestibility (IVDMD), and *in vitro* neutral detergent fiber digestibility (IVNDFD). Addition of tannin or Monensin did not affect ($P > 0.05$) the kinetics parameters. Tannin supplementation reduced ($P < 0.05$) the GP at 24 h compared to Monensin. Addition of Monensin decreased ($P < 0.05$) IVDMD at 96 h and IVNDFD at 48 and 96 h compared to the control. The IVNDFD was lower ($P < 0.05$) with Monensin than with tannin at 48 and 96 h. The NH_3 was lower ($P < 0.05$) with tannin compared with Monensin. By increasing tannin dosage, NH_3 levels changed quadratically ($P < 0.05$). The inclusion of tannin *in vitro* reduced the NH_3 concentration considerably when used in low dose.
Key words: Additive. Beef cattle. Digestibility. Metabolism. Rumen.

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Resumo

O objetivo com este estudo foi avaliar os efeitos de taninos *versus* monensina sobre a fermentação ruminal *in vitro* de uma ração para ovinos em confinamento. Os tratamentos foram: controle (sem a inclusão de aditivos); baixa dose de tanino (2 mg g MS^{-1}); média dose de tanino (4 mg g MS^{-1}), alta dose de tanino (6 mg g MS^{-1}), e monensina (0,02 mg g MS^{-1}). O substrato utilizado foi uma ração para ovinos em confinamento composta por feno e concentrado (15:85 w/w; base da MS). O fluido ruminal foi obtido de três ovinos Santa Inês, machos não castrados, com cânula ruminal. As incubações *in vitro* foram realizadas em quatro semanas consecutivas (uma incubação por semana). A produção de gás (PG) foi mensurada nos tempos 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, 24, 30, 36, 42, 48, 60, 72, 84 e 96 horas de incubação. Nos tempos de 48 e 96 horas, dois frascos/tratamento foram retirados para mensurar pH, concentração de amônia (NH_3), ácidos graxos voláteis (AGV), digestibilidade in vitro da matéria seca (DIVMS) e digestibilidade in vitro da fibra em detergente neutro (DIVFDN). A adição de tanino ou monensina não afetaram ($P > 0,05$) os parâmetros cinéticos. A suplementação de tanino reduz ($P < 0,05$) a PG em 24 horas de incubação comparado com a monensina. A inclusão de monensina reduz ($P < 0,05$) a DIVMS em 96 horas e a DIVFDN em 48 e 96 horas comparada ao controle. A DIVFDN foi menor ($P < 0,05$) com monensina que com tanino em 48 e 96 horas. A NH_3 foi menor ($P < 0,05$) com tanino comparado à monensina. Aumentando a dose de tanino, a NH_3 altera de forma quadrática ($P < 0,05$). A inclusão de tanino reduz consideravelmente as concentrações *in vitro* de NH_3 quando usado em baixas doses.

Palavras-chave: Aditivo. Bovinos de corte. Digestibilidade. Metabolismo. Rúmen.

Introduction

The ionophore Monensin is the most commonly used feed additive in feedlot diets to improve the efficiency of ruminal fermentation (TEDESCHI et al., 2011). Effects include a decrease in the acetate: propionate ratio, improvement of feed efficiency, reduction of rumen deamination, decreases in CH_4 production, and the risks of metabolic diseases (DINIUS et al., 1976this research sought to increase the knowledge around$\\$nthe real-life experience of Jatropha farming in the southern India$\\$nstates of Tamil Nadu and Andhra Pradesh. Launched as an alternative for$\\$ndiesel in India, Jatropha has been promoted as a non-edible plant that$\\$ncould grow on poor soils, yield oil-rich seeds for production of$\\$nbio-diesel, and not compete directly with food production. Through$\\$ninterviews with the farmers, information was gathered regarding their$\\$nsocio-economic situation, the implementation and performance of their$\\$nJatropha plantations, and their reasons for continuing or discontinuing$\\$nJatropha cultivation. Results reveal that 82{%} of the farmers had$\\$nsubstituted former cropland for their Jatropha cultivation. By 2010,$\\$n85{%} (n = 90; RUSSELL; STROBEL, 1989). Despite advantageous benefits of Monensin, growing public concerns over chemical residues in animal-derived foods, threats of antibiotic-resistant bacteria and an outright ban by the European Union (TEDESCHI et al., 2011), the animal feed industry have renewed their interest in exploring the use of safer alternatives in substitution of chemical feed additives historically used for ruminant livestock feeding.

Several natural alternatives such as plants, plant extracts, and secondary metabolites (e.g. tannins, saponins, and essential oils) with antibiotic-like action potential have received considerable attention as natural ways to manipulate ruminal fermentation (MEZZOMO et al., 2011ruminal and total digestibility, ruminal digestion rate, protein efficiency, and microbial efficiency in beef steers fed high concentrate diet (87% of DM; TEDESCHI et al., 2011).

Tannins are secondary plant products and consist of water-soluble polyphenolic compounds with high molecular weight, classified as either condensed or

hydrolysable. Some studies suggest that tannins may improve the use of the dietetic protein without impairing feed intake or carbohydrate digestibility because their phenolic hydroxyl group can bind to the protein or amino acids, protecting from the ruminal tract and therefore prevents enzymatic hydrolysis (WAGHORN et al., 1987). The complexes are presumed to dissociate during the passage through the more acidic environment of the abomasum or in the alkaline environment of the small intestine (MCMAHON et al., 1999). Condensed tannins may also bind directly to hydrolytic enzymes, rendering them catalytically inactive (MAKKAR et al., 1998). In addition, tannins have a potentially wide range of effects on ruminal fermentation, such as the prevention of metabolic disorders (CIESLAK et al., 2012)ammonia concentration ($P<0.001$ and the mitigation of rumen methane emissions (BUENO et al., 2015)zebu beef cattle (Bos taurus indicus. However, these effects on animal metabolism depend on the dose, the substrate, and the type of tannin (GETACHEW et al., 2008).

Therefore, the objective this study was to evaluate the effects of the addition of Monensin and increasing levels of tannins on the kinetics of gas production, dry matter and neutral detergent fiber digestibility, volatile fatty acids concentrations, and ammonia concentrations in feedlot diets.

Materials and Methods

Animal donors of rumen fluids

The experiment was approved by the Institutional Committee for Ethics in the Use of Animals of the UFMT–Cuiabá (244/2015). Three rumen-cannulated male Santa Inês sheep (60±3 kg BW), fed a total mixed ration (TMR) ration with a 40:60 hay: concentrate ratio, previously adapted for 30 days, were used as donors of rumen fluids. The animals were kept in individual sheltered pens equipped with water and feed troughs.

Experimental design and in vitro rumen fermentation

In vitro incubations were carried out over four consecutive weeks (runs). The substrate consisted of a feedlot ration with 15% of *Panicum maximum* cv. Massai hay, 60% ground corn, 10.5% soybean meal, 11.8% soybean hulls, 0.7% urea, and 2% mineral mixture (Table 1).

Table 1. Ingredients and chemical composition of the substrate (DM basis).

Item, % DM	Substrate diet
Ingredients (g kg^{-1})	
Grass hay[a]	148.1
Ground corn	606.3
Soybean meal	104.9
Soy hulls	116.5
Urea	6.21
Mineral mixture	17.7
Chemical composition (g kg^{-1}, DM basis)	
DM	879.6
CP	139.8
NDF	297.7

[a]*Panicum maximum* cv. Massai hay.

A commercial blend of tannin (70% hydrolysable and condensed tannins; Silvafeed-Bypro®, Silvateam-Inudor S.A., Argentina) was used. Experimental treatments were as follows: control (no additives); low tannin (2 mg g DM^{-1}); medium tannin (4 mg g DM^{-1}), high tannin (6 mg g DM^{-1}), and Monensin (0.02 mg g DM^{-1}; Rumensin®Elanco, 20%, USA). The experimental doses of tannin were above and below the manufacturer's recommendation.

To each 120-mL serum bottles, 0.5 g of the base ration was added and the flasks were arranged in a water bath (Dubnoff Agi.Orbital SL-158 Solab). The tannin was diluted in heated water (60 ºC) and the Monensin in ethanol, as described by Ishlak et al. (2015). Both dilutions were performed under anaerobic conditions one day prior to incubation.

Rumen fluid was obtained from each animal before the morning feeding, filtered through cheesecloth with a pore size of 250 µm, and stored in insulated thermos without leaving empty spaces, as recommended by Yáñez-Ruiz et al. (2016). The rumen fluid of the three animals was homogenized and filtered to reduce particle contamination and continuously purged under free-oxygen CO_2 at 39 ºC during the whole manipulation process. Sequentially, 40 mL of buffer solution was added to each bottle according to Goering and Van Soest (1970), followed by 10 mL of rumen fluid, resulting in a rumen fluid: buffer ratio of 1:4 (v/v). Subsequently, 0.2 mL of the diluent-additive was added to each bottle to achieve the final concentration as previously established. Bottles were immediately sealed with rubber caps and aluminum rings and maintained at 39ºC in constant agitation. The gas production volume was recorded at 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, 24, 30, 36, 42, 48, 60, 72, 84, and 96 h of incubation, using the semiautomatic reading technique described by Theodorou et al. (1994) and Mauricio et al. (1999)interfaced with a PC allows accumulated head-space gas pressure values to be directly entered into a spreadsheet. These pressure measurements are then used to generate gas volume estimates using a quadratic function derived from simultaneous pressure and volume measurements. Earlier attempts to derive volume from pressure resulted in predicted volume being under estimated. This discrepancy was most likely due to diffusion of head-space gases into the liquid phase, a factor not considered in Boyle's Law, the initial relationship used. In comparison with the syringe technique (Theodorou et al., 1994.

For each *in vitro* incubation (run), we prepared five glass bottles per treatment, with three bottles to measure gas production over time and two to evaluate *in vitro* DM digestibility (IVDMD) and *in vitro* NDF digestibility (IVNDFD) at 48 and 96 h after the start of the incubation. Three blanks (only ruminal fluid and buffer solution) were also used.

Chemical analysis

Samples of the ration were processed in a 1 mm porosity sieve knife mill and the DM, CP, and NDF analysis procedures were performed according to Detmann et al. (2012) (Table 1). At 48 and 96 h after incubation, two flasks were collected to measure: pH, NH_3, VFA, IVDMD, and IVNDFD.

To determine NH_3 concentration, 2 mL of each vial were sampled at 48 h, centrifuged at 10000 RPM for 10 minutes at temperature of 4 °C, and stored at -20°C for further analysis according to Chaney and Marbach (1962).

To determine the molar ratios of VFA, aliquots of 0.5 µL of the supernatants obtained after centrifugation were injected into the gas chromatograph (Model Shimadzu® GC-2014; Column: VF WAXms-Agilent: 30 m x 0.25 mm x 0.25 µm, run time of 14 minutes). Based on the concentrations of acetic, propionic, and butyric acids and the ppm/molar ratio of each acid, the appropriate analyses were performed with mmol/L versions.

To evaluate IVDMD and IVNDFD, the withdrawn vials were immediately placed in cold

water to stop the fermentation. The pH was measured and the remaining contents of each vial were placed in Ankom® F57 bags (Ankom Technology Corp., Macedon, NY), oven-dried at 105 °C for 16 h, and weighed. Subsequently, the bags were placed in the Ankom^{200} Fyber Analyzer with neutral detergent solution (VAN SOEST et al., 1991) using a heat-stable α-amylase, omitting sodium sulfite and kept at 100 °C for 1 h. The Ankom® F57 bags were then dried in a circulation oven at 55 °C for 24 h, at 105 °C for 1 h, and weighed.

Calculations and statistical analyses

The parameters of the dynamics of *in vitro* gas production over time were estimated using the NLIN procedure of SAS (version 9.3), following the Gompertz function (SCHOFIELD et al., 1994) measured by computer-interfaced pressure sensors, was used to follow the digestion of a crystalline processed cellulose, a bacterial cellulose, and mixtures of these substrates by mixed ruminal bacteria. A first-order, substrate limited model (simple exponential with lag:

$$\hat{Y}_{ijk} = \mu + B_i + M_j + S_k + MS_{jk} + \varepsilon_{ijk}$$

where GP is the cumulative gas production (mL), *a* is the theoretical maximum of gas production (mL), *b* is the maximum gas production rate (mL h^{-1}) that occurs at the point of inflection of the curve, *L* is the lag time (h) defined as the time-axis intercept of a tangent line at the point of inflection, and *t* is the incubation time (h). We used the interactive process of the Marquardt algorithm for adjustments.

The variables were analyzed using the MIXED procedure of SAS (version 9.3). Prior to statistical analysis, data of each of the four runs within the same treatment were averaged. Means values of each run were used as the experimental unit (UDÉN et al., 2011). The statistical model included treatments as a fixed factor. Contrasts were generated to compare Monensin with control (without tannin inclusion) and Monensin with tannin levels (2, 4, and 6 mg g DM^{-1}). Orthogonal contrasts were used to partition specifically the effects of tannin levels (0, 2, 4, and 6 mg g DM^{-1}) on linear and quadratic using the CONTRAST statement of SAS. The LSMEANS option was used to generate individual means for each treatment. In all analyses, significance was declared at ($P < 0.05$).

Results

The inclusion of the additives did not affect ($P > 0.05$) the kinetic parameters of the fermentation. Tannin reduced ($P < 0.05$) GP at 24 h compared to Monensin (Table 2).

Monensin decreased ($P < 0.05$) IVDMD at 96 h and IVNDFD at 48 and 96 h compared to the control (Table 3). The inclusion of Monensin also reduced ($P < 0.05$) the IVNDFD at 48 and 96 h compared to tannin (Table 3).

The inclusion of tannin or Monensin did not affect ($P > 0.05$) the total VFA concentration, and there was also no change ($P > 0.05$) in the acetate: propionate ratio (Table 4). The NH_3 concentration was lower ($P < 0.05$) with the inclusion of tannin when compared to Monensin (Table 4). There was a quadratic effect ($P < 0.05$) of tannin in NH_3 (Table 4). The pH was not affected ($P > 0.05$) by the inclusion of Monensin or tannin in the diets (Table 4).

Discussion

Changes in ruminal fiber digestion and gas production usually occur as a function of the ability of tannins to form complexes with the protein, with adverse effects on the fermentation process and the production of short chain fatty acids (BUENO et al., 2015)zebu beef cattle (Bos taurus indicus. Commonly, fiber digestibility and gas production are reduced with the inclusion of both condensed and hydrolysable tannin (BHATTA et al., 2009; BUENO et al., 2015;zebu beef cattle (Bos taurus

indicus FRUTOS et al., 2004) . Possibly, this occurs due to the interference of tannins in cell wall degradation by the inhibition of microbial enzymes and adhesion of fibrolytic populations, thereby decreasing the concentration in gas production of the rumen (BUENO et al., 2015)zebu beef cattle (Bos taurus indicus.

However, the inclusion of tannin did not reduce fiber digestion and thus, the lower gas production observed over a period of 24 h was not maintained throughout the incubations. A reason for this effect would be the possibility of adaptation of the microorganisms to the presence of the tannins throughout the incubation (ANASSORI et al., 2012).

Ionophores, such as Monensin, change the ion movement across the membranes of gram-positive bacteria, altering the proton gradient and pH within the cell, which results in lysis of the bacteria (MARTIN, 1998). Thus, the growth of gram-positive bacteria is inhibited by Monensin, especially fibrolytic bacteria (e.g. *Ruminococcus albus, Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens*) (CHEN; WOLIN, 1979), thus causing a reduction in fiber digestibility as observed.

Table 2. In vitro fermentation and total gas production as a function of additive type.

Variables	TANNIN (g/kg DM)				MON	SEM[d]	CONTRASTS[e]			
	0	2	4	6			MON* 0	MON* TAN	TAN L	TAN Q
a[a]	142.78	143.59	141.80	141.83	139.34	2.537	0.352	0.312	0.687	0.879
b[b]	0.073	0.074	0.074	0.073	0.079	0.003	0.125	0.088	0.936	0.880
L[c]	2.27	2.32	2.34	2.38	1.96	0.249	0.405	0.206	0.755	0.975
Gas Production 6 h	19.62	19.56	19.69	18.94	21.22	0.807	0.181	0.070	0.608	0.676
Gas Production 12 h	41.91	42.27	42.27	41.61	44.79	1.136	0.094	0.054	0.861	0.663
Gas Production 24 h	80.78	81.30	80.46	78.58	87.12	2.194	0.059	0.014	0.461	0.593
Gas Production 48 h	127.09	126.41	127.41	124.10	124.92	1.937	0.440	0.644	0.371	0.507
Gas Production 72 h	139.86	140.44	139.56	137.35	139.03	2.036	0.778	0.971	0.371	0.503
Gas Production 96 h	146.24	146.88	145.81	143.83	142.32	2.477	0.281	0.282	0.466	0.604

[a]*a* is asymptotic gas production (mL/g DM); [b]*b* is the rate of digestion (h^{-1}); [c]*L* is the *Lag time* (h). [d]SEM is the standard error mean. [e]Contrasts: MON vs 0 (Monensin vs Substrate without additive); MON vs TAN: (Monensin vs average tannin levels 2, 4, and 6 mg g); TAN L: (Linear tannin 0, 2, 4, and 6), and TAN Q: (Quadratic tannin 0, 2, 4, and 6).

Table 3. In vitro digestibility of DM and NDF as a function of the inclusion of Monensin and tannin content.

Variables	TANNIN (g/kg DM)				MON	SEM[c]	CONTRASTS[d]			
	0	2	4	6			MON* 0	MON* TAN	TAN L	TAN Q
IVDMD[a] 48 h	76.00	73.68	75.21	73.73	72.85	2.185	0.325	0.600	0.596	0.850
IVDMD 96 h	82.49	82.93	82.97	83.30	79.82	0.875	0.047	0.006	0.540	0.953
IVNDFD[b] 48 h	54.18	52.35	53.15	53.22	43.58	2.387	0.007	0.004	0.850	0.698
IVNDFD 96 h	73.07	73.08	72.79	72.76	69.20	0.987	0.014	0.006	0.785	0.982

IVDMD[a] is the *in vitro* dry matter digestibility (%), IVNDFD[b] is the *in vitro* digestibility of neutral detergent fiber (%), [c]SEM is the standard error mean. [d]Contrasts: MON vs 0 (Monensin vs Substrate without additive); MON vs TAN: (Monensin vs average tannin levels 2, 4, and 6 mg g); TAN L: (Linear tannin 0, 2, 4, and 6), and TAN Q: (Quadratic tannin 0, 2, 4, and 6).

Table 4. Effect of the use of Monensin or tannin content on the concentrations of VFA and ammonia and on pH values of the medium fermentation.

Variables	TANNIN (g/kg DM)				MON	SEM[d]	CONTRASTS[e]			
	0	2	4	6			MON* 0	MON* TAN	TAN L	TAN Q
Acetate	5.30	4.96	5.00	4.91	4.77	0.576	0.530	0.788	0.673	0.825
Propionate	4.23	4.26	4.32	4.32	4.38	0.665	0.877	0.923	0.909	0.983
Butyrate	3.73	3.90	4.07	4.23	4.23	0.964	0.720	0.885	0.709	0.991
Acetate: Propionate	1.26	1.18	1.18	1.16	1.13	0.104	0.380	0.722	0.518	0.755
VFA Total[a]	13.26	13.12	13.39	13.46	13.38	2.056	0.967	0.981	0.925	0.960
NH_3-N[b]	14.41	12.17	12.76	12.97	15.12	0.349	0.171	<0.001	0.030	0.003
pH[c] 48	6.58	6.59	6.64	6.67	6.63	0.102	0.709	0.978	0.491	0.971
pH 96	6.64	6.63	6.64	6.62	6.60	0.052	0.604	0.630	0.818	0.914

[a]VFA is the volatile fatty acids (mmol/L); [b]NH_3-N is the ammoniacal nitrogen (mg/dL); [c]pH 48 and 96 is the pH at 48 and 96 hours; [d]SEM: Standard error mean. [e]Contrasts: MON vs 0 (Monensin vs Substrate without additive); MON vs TAN: (Monensin vs average tannin levels 2, 4, and 6 mg g); TAN L: (Linear tannin 0, 2, 4, and 6), and TAN Q: (Quadratic tannin 0, 2, 4, and 6).

The reduction in fiber digestion may cause an alteration in the production of VFA's, especially acetate, altering the acetate: propionate ratio (BUENO et al., 2015)zebu beef cattle (Bos taurus indicus. Although a lower digestibility of NDF was observed with Monensin, no differences were found for VFA. In addition, gas production is an indicative of the quantitative production of VFA (KIM et al., 2014)each surgically fitted with a ruminal cannula, consuming 0.50 alfalfa cubes and 0.50 cracked corn-based concentrate at 1.75??NEm requirements were used as rumen fluid donors. For both experiments in vitro gas production was measured in a completely random design with a 3??2??3 factorial treatment structure. Factors were diet [control (no substrate. Therefore, as gas production was similar between additives, the absence of effects on VFA could also be expected.

Considering the lower NH_3 concentrations obtained with tannin, it is suggested that lower levels of protein were degraded in the medium, as a result of tannin affinity for the protein. Additionally, condensed tannins may inhibit the activity of protozoa that degrade proteolytic bacteria (BHATTA et al., 2009; MCMAHON et al., 1999). This mode of action against microorganisms is still not well understood (BODAS et al., 2012). In this sense, it remains to be determined whether the reduction in protein use associated with tannin is a result of protein protection or direct inhibition of proteolytic bacteria (MCMAHON et al., 1999).

Although the concentration of NH_3 was lower at all levels of tannin inclusion, it was observed that the lower concentration was obtained with the lower dose used and that as the tannin inclusion was increased, the NH_3 concentration also increased. This demonstrates that there may be other mechanisms involved besides the affinity between tannins and protein.

In addition, factors such as ration composition, adaptation period and exposure to the product, the way the samples were collected, the type and concentrations of the additives in the rations (BODAS et al., 2012; GETACHEW et al., 2008; MCGUFFEY et al., 2001), and the donor animal of the inoculum (BUENO et al., 2015; FRUTOS et al., 2004)zebu beef cattle (Bos taurus indicus are probably related to the variability of the literature

results regarding the effects of Monensin and tannin on the digestibility of DM, NDF, and proportions of VFA and NH_3.

Conclusion

The inclusion of tannin *in vitro* reduced the NH_3 concentration at all the dosages used, being more considerably when used in low dose. *In vivo* studies are needed to validate these effects.

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