

Increasing inclusions of *Tribulus terrestris* as an additive in diets of sheep kept in confinement

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[Inclusões crescentes de *Tribulus terrestris* como aditivo em dietas para ovinos confinados]

Y.S. Schunk¹ , C.F.M. Mendes² , R.A. T. Almeida¹ , I.P. Raimundo² , I.Q. Takahashi² ,
C.F. Silva² , R.L. Valença³ , R.O. Rego³ , L.C. Nunes³ , M.T.C. Almeida³ 

¹ Graduate, Universidade Federal do Espírito Santo, Alegre, ES, Brasil

² Undergraduate, Universidade Federal do Espírito Santo, Alegre, ES, Brasil

³ Universidade Federal do Espírito Santo, Alegre, ES, Brasil

ABSTRACT

The aim was to evaluate the effects of increasing levels of *Tribulus terrestris* (TT) in diets of sheep in confinement on rumen parameters and blood metabolites. The experiment was conducted in two stages, *in vitro*, with inclusions ranging from 0 to 15.0 g/kg of TT in dry matter of the diet and the *in vitro* digestibility of dry matter and nutrients, pH, measurement of ammoniacal nitrogen and greenhouse gases were determined. And *in situ*, with inclusions of 0 to 5.0g/kg, in which five cannulated sheep were distributed in a 5 × 5 Latin square design to evaluate fermentation parameters, protozoan populations and blood metabolic profile. The inclusion of TT, regardless of dosage, resulted in decrease on *in vitro* digestibility, with quadratic effect on dry matter and nutrients, and reduction in ammoniacal nitrogen and methane. In terms of fermentation parameters, the safest dosages *in vitro* were 1.25 and 2.5g/kg. Inclusion of up to 5.0g/kg did not affect fermentation parameters *in situ*, did not harm the blood metabolic profile and reduced the population of small protozoa. Studies with inclusions greater than 5.0g/kg should be carried out to confirm the effect on protozoa, with subsequent analysis of production performance.

Keywords: ammonia, methane, protozoa, ruminants, saponins

RESUMO

O objetivo deste estudo foi avaliar os efeitos de inclusões crescentes de *Tribulus terrestris* (TT) em dietas para ovinos confinados, sobre parâmetros ruminais e metabólitos sanguíneos. O experimento foi conduzido em duas etapas: *in vitro*, com inclusões que variaram de 0 até 15,0g/kg de TT na matéria seca da dieta, sendo determinadas as digestibilidades *in vitro* da matéria seca e dos nutrientes, do pH, da mensuração de nitrogênio amoniacal e dos gases de efeito estufa; e *in situ*, com inclusões de 0 até 5,0g/kg, no qual cinco ovinos canulados foram distribuídos em um delineamento de quadrado latino 5 × 5. Foram avaliados parâmetros fermentativos, populações de protozoários e perfil metabólico sanguíneo. A inclusão de TT, independentemente da dosagem, proporcionou redução na digestibilidade *in vitro*, com efeito quadrático sobre matéria seca e nutrientes, diminuições do nitrogênio amoniacal e metano. Em termos de parâmetros fermentativos, as dosagens mais seguras *in vitro* foram 1,25 e 2,5g/kg. A inclusão de até 5,0 g/kg não afetou os parâmetros fermentativos *in situ*, não prejudicou o perfil metabólico sanguíneo e reduziu a população de pequenos protozoários. Estudos com inclusões maiores que 5,0g/kg devem ser efetuados para confirmar o efeito sobre os protozoários, com posteriores análises de desempenho produtivo.

Palavras-chave: amônia, metano, protozoários, ruminantes, saponinas

INTRODUCCION

The feeding of ruminants has intensified over the years due to population growth and the resulting greater demand to meet the market. This has led

to investments in genetic improvement, animal management, and nutrition. Among these technologies are feed additives, which aim to increase the efficiency of diet use and promote animal growth, optimizing production rates (Vieira *et al.*, 2020).

Corresponding author: yasmini.schunk@hotmail.com

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The primary additives utilized in ruminant diets are ionophores. These antimicrobials are used as growth promoters, enhancing nutrient utilization and increasing the animal's rate of live weight gain by selectively modulating the rumen microbiota (Nicodemo, 2001). However, the increasingly frequent use of these substances in ruminant feed is a major risk factor for cross-resistance to antimicrobials (Percio *et al.*, 2019). For this reason, in 2006 the European Union banned the use of antibiotics as growth promoters in animal feed (Arowolo and He, 2018).

In this context, it is necessary to search for new alternatives to the use of antimicrobials in ruminant feed to achieve the benefits of rumen modulation and ensure the health and well-being of animals and humans (Percio *et al.*, 2019). Potential substitutes include natural additives such as vegetable oils and extracts (Soltan *et al.*, 2018; Orzuna-Orzuna *et al.*, 2021). *Tribulus terrestris* is a plant belonging to the *Zygophyllaceae* family, rich in medicinally important constituents, such as flavonoids, glycosides, alkaloids and steroid saponins, the latter being mainly responsible for the biological activity of products derived from this plant (Wang *et al.*, 2016).

Saponins offer several benefits in ruminant nutrition, they form a complex with proteins, which reduces protein degradation in the rumen and increases protein flow into the duodenum (Kholif, 2023); controls the development of ciliated protozoa in the rumen (Liu *et al.*, 2019), reducing their contribution to methane production (Rira *et al.*, 2015); microbial protein synthesis is improved due to reduced bacterial predation by protozoa (Souza *et al.*, 2016).

In view of the need to reduce the use of antimicrobials in ruminant feed and to find alternatives to the use of ionophores, the aim of this study was to evaluate the effects of increasing inclusions of *Tribulus terrestris* dry extract, *in vitro* and *in situ*, in the diet of confined sheep, on rumen parameters and blood metabolic profile, in order to find a safe dosage for use in the diet of confined sheep.

MATERIAL AND METHODS

The project's experimental procedures were approved by the Ethics Committee for the Use of Animals at the Alegre Campus of UFES (Ceua - Alegre), under protocol number 005/2021, in accordance with the rules of the National Council for the Control of Animal Experimentation.

The experiment was conducted in two stages. The first stage involved an *in vitro* test using increasing amounts of the dry extract of TT (40% saponins): 0 (control), 1.25, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0g/kg in dry matter (DM) of the total diet. The rumen fluid used for the *in vitro* incubations was obtained from two donor sheep, Santa Inês × Dorper crossbreds, that were healthy and fitted with a rumen cannula. The effects on the *in vitro* digestibility of dry matter (IVDDM), neutral detergent fiber (IVDNDf), acid detergent fiber (IVDADF), crude protein (IVDCP), production of greenhouse gases methane (CH₄) and carbon dioxide (CO₂), and rumen fermentation parameters (pH and ammonia nitrogen) were evaluated.

The second stage was carried out *in situ*, using inclusions of 0, 1.25, 2.5, 3.75 and 5.0g/kg which corresponded to the five experimental treatments, in which five male sheep, healthy, uncastrated, Santa Ines × Dorper crossbreds, weighing from 40 kg and fitted with a rumen cannula were distributed in a 5 × 5 Latin square design, to assess pH, ammonia nitrogen concentration, protozoa population and blood metabolic profile.

This stage lasted 75 days, with five experimental periods, each lasting 15 days. At the end of each period, rumen fluid was collected, starting at 7 a.m. and ending at 7 p.m., with two-hour intervals between collections. The experimental diets for both stages consisted of corn silage, cornmeal, soybean meal and calcitic limestone (Table 1), with a 30:70 volume:concentrate ratio.

The *in vitro* digestibility was assessed using an adaptation of the Ankom® technique (2023), in which samples of the experimental diet containing TT inclusions were placed in polyester filter bags (F-57, Ankom®) for digestion in a rotary incubator (model NL-162-02, New Lab).

Dry matter (DM) was analyzed using AOAC method 967.06 (Official..., 1990), which involves pre-drying and final oven drying. The Van Soest (1967) method, as described by Silva and Queiroz (2002), was used to determine neutral detergent fiber (NDF) and acid detergent fiber (ADF), using neutral and acid detergent solutions, respectively. Crude protein (CP) was measured by determining nitrogen using the semi-micro Kjeldahl process (Official..., 2002) with nitrogen distillation equipment (SL-74 Nitrogen Distiller, Solab).

To assess greenhouse gases, 50mL glass vials were used, in which 0.2 g of the experimental diet, 10mL of rumen fluid and 20 mL of McDougall solution were added and the gases produced during the incubation periods of 0, 3, 6, 9, 12, 24, 36 and 48 hours were packed in vacuum tubes and analyzed in a gas chromatograph (Shimadzu Green House Gas Analyzer GC-2014; Kyoto, Japan).

Table 1. Ingredients and nutritional composition of the experimental diets.

Ingredients	%, DM
Corn silage	43.75
Soybean meal	22.00
Cornmeal	33.45
Calcitic limestone	0.80
Calculated nutrient composition	
DM, %	65.08
CP, % DM	17.60
ME, Mcal/kg DM	2.77
EE, % DM	3.65
NDF, % DM	27.31
Ca, % DM	0.51
P, % DM	0.33

DM= dry matter; CP= crude protein; ME= metabolizable energy; EE= ether extract; NDF= neutral detergent fiber; Ca= calcium; P= phosphorus.

To evaluate rumen fermentation parameters, a portable pH meter with automatic temperature compensation (K39-0014PA, Kasvi) was used to measure pH during both the *in vitro* and *in situ* phases. Ammonia nitrogen levels were determined using the INCT-CA method N-007/1 (Detmann *et al.*, 2012).

The protozoa were assessed by placing a drop of rumen fluid between a slide and a coverslip and observing it under a microscope, in which the

protozoa populations were classified as small, medium and large, and the intensity of their motility was also assessed. The metabolic blood profile analyses were conducted using blood samples collected in tubes without anticoagulant. Commercial biochemical kits (BioTécnica) were used to measure the metabolites. The procedure was carried out in a semi-automated biochemical analyzer (model Bio-2000 IL, Bioplus).

The normality of residuals in the *in vitro* stage data was tested using the Shapiro - Wilk test, with the PROC UNIVARIATE procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC). Homogeneity of variances was tested using the PROC TRANSREG 'BOXCOX' procedure (SAS 9.4). Both stages' data were analyzed using the PROC MIXED package of the SAS statistical program version 9.4, with a significance level of 5%.

RESULTS

The increasing inclusion of TT in diets for confined sheep had a quadratic effect on IVDDM, IVDNDF, IVDADF and IVDGP, with the dosages of 5.0, 7.5 and 10.0g/kg having the lowest digestibility, while the dosages of 1.25 and 2.5g/kg had the highest values when compared to the other treatments with added TT (Table 2; $p < 0.05$). When comparing the average of all the treatments containing TT against the control treatment (Cont. $\times$ TT), this reduction in digestibility is confirmed, with decreases of 18.92% being noted for IVDDM; 10.07% for IVDNDF; 25.14% for IVDADF and a reduction of 34.35% IVDGP (Table 2; $p < 0.05$).

The inclusion of TT in N-NH₃ resulted in a reduction that followed a quadratic behavior, with the lowest values observed for treatments containing 7.5, 10.0, and 15.0g/kg (Table 2; $p < 0.05$). A reduction of 20.73% was observed when comparing the average of diets containing TT (7.927mg/dL) with the control diet (Table 2; $p < 0.05$). The pH values did not show significant variations around the average (Table 2; $p < 0.05$).

In terms of *in vitro* gas production, the total volume of gases and CO₂ exhibited linear reductions (Table 3; $p < 0.05$). However, when comparing all treatments containing TT to the control (Cont. $\times$ TT), no significant difference was observed. Regarding CH₄, all treatments

containing TT showed a reduction compared to the control (Cont. × TT; Table 3; $p < 0.05$). The maximum reduction of 21.4% was achieved with the inclusion of 7.5g/kg of the extract (Table 3).

Regarding *in situ* fermentation parameters and rumen protozoan populations, there was no interaction between collection time × treatments

for the variables pH, N-NH₃ and populations of medium and large protozoa, so the average of all the times for each treatment was evaluated (Table 4; $p > 0.05$). For small protozoa there was a linear reduction ($p < 0.05$), however, when comparing all the treatments with TT against the control (Cont. × TT), there was no difference (Table 4; $p > 0.05$).

Table 2. *In vitro* digestibility of DM and nutrients, final pH and concentration of ammoniacal nitrogen (N-NH₃) of diets containing increasing inclusion of *Tribulus terrestris* for confined sheep

Item	g/kg DM of <i>Tribulus terrestris</i>									p-value		
	0	1.25	2.5	5.0	7.5	10.0	12.5	15.0	SEM	L.	Q.	Cont X TT
IVDDM	73.63	67.82	68.34	51.30	51.51	52.99	64.63	61.31	1.054	<.0001	<.0001	<.0001
IVDNDf	70.56	68.99	69.74	57.80	56.45	60.28	67.38	63.61	0.722	<.0001	<.0001	<.0001
IVDADF	49.98	59.09	51.82	23.36	19.58	26.05	42.17	39.90	1.606	<.0001	<.0001	<.0001
IVDCP	82.75	61.87	65.81	49.89	48.50	39.57	58.33	56.36	1.576	<.0001	<.0001	<.0001
pH	6.30	6.32	6.30	6.36	6.38	6.375	6.4	6.4	0.009	<.0001	0.678	0.0053
N-NH ₃ *	10.00	8.568	8.28	7.99	7.568	7.675	7.85	7.56	0.167	<.0001	0.004	<.0001

DM= dry matter; SEM= standard error of the mean; Cont.= Control; TT= *Tribulus terrestris*; IVDDM= *in vitro* digestibility of dry matter; IVDNDf= *in vitro* digestibility of neutral detergent fiber; IVDADF= *in vitro* digestibility of acid detergent fiber; IVDCP= *in vitro* digestibility of crude protein; N-NH₃= ammonia nitrogen; L.= linear; Q.= quadratic.

* Unit of measurement in mg/dL.

Table 3. *In vitro* gas production in diets for confined sheep containing increasing amounts of *Tribulus terrestris*

Tribulus terrestris												
Item	g/kg DM of <i>Tribulus terrestris</i>									p-value		
	0	1.25	2.5	5.0	7.5	10.0	12.5	15.0	SEM	L.	Q.	Cont. X TT
TV ¹	12.1	12.0	12.2	12.0	11.1	11.9	11.5	11.7	0.237	0.010	0.314	0.107
CH ₄ [*]	10.8	9.93	9.17	8.82	8.49	9.03	8.77	8.92	0.191	<.0001	<.0001	<.0001
CO ₂ [*]	41.1	41.3	41.5	37.9	36.3	38.5	37.7	38.5	0.810	0.011	0.168	0.124

TV= Total volume; SEM= standard error of the mean; L.= Linear; Q.= Quadratic; Cont.= Control; TT= *Tribulus terrestris*. ¹ Unit of measurement in mL. * Unit of measurement in mL/g.

Table 4. *In situ* evaluation of pH, N-NH₃ and populations of protozoa (small, medium and large) in the rumen fluid of confined sheep fed a diet containing an increasing inclusion of *Tribulus terrestris* as additive

Item	g/kg DM of <i>Tribulus terrestris</i>					p-value			
	0	1.25	2.5	3.75	5	SEM	L.	Q.	Cont. × TT
pH	6.36	6.20	6.30	6.27	6.73	0.023	0.448	0.227	0.092
N-NH ₃ *	10.48	9.80	10.66	11.08	9.68	0.252	0.260	0.297	0.956
S	0.45	0.41	0.37	0.36	0.33	0.010	0.038	0.869	0.331
M	0.47	0.50	0.37	0.51	0.52	0.011	0.934	0.563	0.893
L	0.08	0.08	0.09	0.13	0.10	0.005	0.147	0.435	0.449

S= population of small protozoa; M= population of medium protozoa; L= population of large protozoa; SEM= standard error of the mean; L.= linear; Q.= quadratic; Cont.= control; TT= *Tribulus terrestris*. * Unit of measurement in mg/dL.

Regarding the blood metabolic profile, a quadratic effect on the total protein (PROT) and cholesterol variables was observed with the addition of TT. The treatment with the addition of 2.5g/kg resulted in the highest PROT value and the lowest cholesterol value (Table 5; $p < 0.05$).

The average reduction in cholesterol was 14.99% (Cont. $\times$ TT; Table 5) when comparing treatments containing TT (48.63mg/dL) to the control treatment (57.20mg/dL). A linear decrease in creatine kinase (CK) was observed with the inclusion of TT ($p < 0.05$). No significant differences were observed between treatments for the other measured metabolites (Table 5).

Table 5. Blood metabolic profile of feedlot sheep fed a diet containing increasing amounts of *Tribulus terrestris* as additive

Item	Ref. Values ¹	g/kg DM of <i>Tribulus terrestris</i>						p-value		
		0	1.25	2.5	3.75	5	SEM	L.	Q.	Cont. $\times$ TT
PROT (g/L)	6.0 - 7.9	5.90	5.90	6.26	5.98	5.57	0.102	0.252	0.023	0.859
Alb. (g/L)	2.6 - 4.2	3.64	3.52	3.40	3.36	3.25	0.100	0.103	0.873	0.175
Glob. (g/L)	3.5 - 5.7	2.26	2.38	2.86	2.62	2.32	0.160	0.641	0.096	0.277
Ur. (mg/dL)	17 - 43	32.52	29.82	30.38	37.10	28.87	2.030	0.999	0.773	0.842
Cr. (mg/dL)	1.2 - 1.9	1.14	1.08	1.08	1.14	1.07	0.029	0.700	0.867	0.500
Chol. (mg/dL)	52 - 76	57.20	50.20	46.40	47.60	50.32	1.652	0.085	0.033	0.013
Trig. (mg/dL)	15 - 57	30.80	29.40	19.80	21.40	27.00	2.302	0.294	0.155	0.207
AST (U/L)	<280	56.80	53.00	53.40	57.00	62.20	2.519	0.303	0.211	0.932
CK (U/L)	<40	22.80	33.20	26.40	19.40	16.17	2.125	0.046	0.076	0.811
P (mg/dL)	5.0 - 7.3	9.30	6.76	6.93	6.96	6.84	0.521	0.198	0.265	0.060
Mg (mg/dL)	2.2 - 2.8	3.32	3.22	3.12	3.30	3.01	0.124	0.559	0.935	0.608

PROT= total proteins; Alb.= albumin; Glob.= globulins; Ur.= urea; Creat.= creatinine; Chol.= cholesterol; Trig.= triglycerides; AST= aspartate aminotransferase; CK= creatine kinase; P= phosphorus; Mg= magnesium; SEM= standard error of the mean; L.= linear; Q.= quadratic; Cont.= control; TT= *Tribulus terrestris*. ¹Ref. values= reference values for sheep: González and Silva (2022).

DISCUSSION

The decrease in IVDDM, IVDNDF, and IVDADF (Table 2) may be attributed to the saponins' ability to impede the growth of certain rumen cellulolytic bacteria (gram-positive), thereby hindering the breakdown of fibrous carbohydrates and adversely affecting the digestion of NDF, ADF, and DM. Mao *et al.* (2010) observed a reduction in the population of gram-positive *Ruminococcus flavefaciens* when saponins were added to a medium containing microbial populations. However, there was no reduction in the population of gram-negative *Fibrobacter succinogenes*.

Although saponins have a greater predilection for acting on rumen protozoa, due to their high affinity for forming complexes with the sterols in the plasma membrane of these microorganisms, saponins can also act on gram-positive rumen bacteria, because the structure of the cell membrane is less complex than that of gram-negative bacteria (which, in addition to the

plasma membrane, also have an outer membrane with porin channels), making them more susceptible to the action of saponins, which causes a change in osmolarity and cell lysis in these microorganisms (Souza *et al.*, 2016).

The lower IVDGP (Table 2) may be due to a lack of synchronization between carbohydrate and protein fermentation. The synthesis of microbial protein in the rumen depends on the fermentation of carbohydrates at the rumen level. For the rumen bacteria to assimilate the ammonia from the fermentation process and form microbial protein, they combine the ammonia with carbon skeletons from the digestion of carbohydrates, thus incurring energy expenditure (Medeiros and Marino, 2015). With the reduction in DM digestibility noted in this study, the peak energy available for bacterial growth only occurred after the peak ammonia production, affecting microbial protein synthesis and interfering with IVDGP (Guimarães Junior *et al.*, 2016).

The decrease in N-NH₃ concentration observed in this study (Table 2) support the findings of Jadhav *et al.* (2018), who observed a reduction in N-NH₃ of up to 35% when evaluating the *in vitro* effects of *Camellia sinensis* saponins on rumen fermentation. This could be related to the impact of saponins on reducing protozoa populations. Protozoa can contribute between 10% and 40% to the rumen concentration of nitrogenous compounds due to their predatory action on rumen bacteria (Kholif, 2023).

It is estimated that protozoa can digest around 0.1% of rumen bacteria per minute, contributing to an increase in the concentration of ammonia nitrogen, so controlling the development of these microorganisms in the rumen helps to avoid an excess of this nitrogenous compound (Solomon *et al.*, 2022). In this study, although there was a reduction in the concentration of N-NH₃, this would not be a limiting factor for the bacteria to grow, as the values in all treatments were above the minimum required for sheep, which is 5.0mg/dL (Furtado *et al.*, 2014).

The *in vitro* pH values in this study were within the range considered ideal for maintaining the activity of rumen cellulolytic bacteria (Table 2), which is 6.2 to 6.7 (Oliveira *et al.*, 2013). This is different from what was observed by Freitas *et al.* (2018), who found a decrease in pH to 5.43 in 24 hours of incubation when they added 250 g/L of *Urochloa humidicola* extract. Therefore, it can be concluded that the doses of TT extract used in this study did not affect the ideal pH range for the cellulolytic bacteria to act *in vitro*.

Regarding the production of greenhouse gases *in vitro*, the reduction observed for CH₄ (Table 3) is consistent with the findings of Jadhav *et al.* (2018), who also found a reduction in this gas when evaluating the effects of *Camellia sinensis* saponins. One of the reasons for this reduction of CH₄ *in vitro* is related to the suppressive effect of saponins on protozoa, as these microorganisms can contribute up to 25% of rumen methane production by forming a symbiotic relationship with methanogenic archaea, providing hydrogen ions for these microorganisms to use in the reaction to reduce carbon dioxide to methane (Li and Powers, 2012).

Another factor that should be considered is that some plant extracts have adverse effects on

digestibility by helping to reduce ruminal CH₄ production, such as saponins, which interfere with the breakdown of fibrous carbohydrates by suppressing the development of some gram-positive cellulolytic bacteria, which, by hydrolyzing cellulose, make CO₂ and H₂ available for methanogenic archaea to produce CH₄, consequently reducing CH₄ production, since there will be less CO₂ and H₂ available for methanogenesis (Medjekal *et al.*, 2017).

In the *in situ* test, a reduction in protozoan populations was expected with the inclusion of TT, but this effect only occurred for the small protozoan population (Table 4). The lack of effect on the other populations may be related to the fact that this is an *in situ* test where there is a constant renewal of substrates for fermentation and consequently for the proliferation of microbial populations in the rumen environment, which is different from *in vitro* tests where there is no renewal of substrates, as shown by Guo *et al.* (2008), who worked with a dosage of 0.4 mg/ml of saponin *in vitro* and observed a 51% decrease in the protozoan count.

For the variables pH and N-NH₃ evaluated *in situ*, they remained within the values considered suitable for sheep (Table 4). In the case of pH, the ideal range for the action of cellulolytic bacteria is 6.2 to 6.7 (Van Soest, 1994; Oliveira *et al.*, 2013) and for N-NH₃, the minimum concentration for microbial development is 5.0mg/dL (Furtado *et al.*, 2014), so pH and N-NH₃ were not limiting for bacterial activity and development *in situ* in this study.

Regarding the blood metabolic profile, even with the increase in PROT concentration at 2.5 g/kg (Table 5), the value remained close to the reference range considered ideal for sheep, which according to Kaneko *et al.* (2008) is 6.0 to 7.9g/L, indicating that the inclusion of TT did not adversely affect the serum level of this metabolite.

For cholesterol, the reduction observed agrees with other authors, who also identified a decrease in the level of this metabolite in blood serum when investigating the effects of TT on poultry of the *Numida meleagris* species (Christev *et al.*, 2011) and on Plymouth Rock roosters (Grigorova *et al.*, 2008). This is because protodioscin, a furostanol-type saponin found in

TT, can increase the release of luteinizing hormone (LH), which stimulates testosterone production in the Leydig cells (Gauthaman and Ganesan, 2008; Grigorova *et al.*, 2008). The increased production of testosterone lowers cholesterol levels, as cholesterol is used in the synthesis of steroid hormones and acts as a precursor to sex hormones (Christev *et al.*, 2011).

For the activity of the CK enzyme, in this study, the enzyme did not exceed the recommended values for the species (Table 5), remaining below 40 U/L (González and Silva, 2022). The increase in CK can occur in cases of TT intoxication, as reported by Taghavi and Nourani (2018) in a case of natural intoxication by the plant in sheep, the authors associated the increase in CK enzyme activity with the toxic action of excess saponins on the cardiac musculature. For the present study, it can be inferred that there was no damage to the muscle fibers due to the use of TT.

Regarding the mineral profile, the study's findings indicate that the inclusion of TT did not affect the absorption and function of phosphorus and magnesium (Table 5). Adequate levels of phosphorus aid in the development of rumen cellulolytic bacteria, which require more of this mineral for their growth and metabolism. Additionally, a portion of this mineral is present in saliva and helps to buffer rumen pH. Magnesium acts as a cofactor for enzymes involved in the metabolism of proteins, lipids, and carbohydrates, as well as contributing to the proper functioning of nerve and muscle cells. A deficiency of this mineral can lead to tetany, while excess is excreted via urine (González and Silva, 2017).

CONCLUSIONS

The dry extract of *Tribulus terrestris* showed to modulate the rumen environment by reducing the *in vitro* digestibility of dry matter and nutrients, as well as the production of CH₄ and N-NH₃, with dosages of 1.25 and 2.5g/kg being the safest in terms of fermentation parameters. The inclusion of up to 5.0g/kg of the extract reduced the population of small protozoa without compromising the fermentation parameters *in situ* and the blood metabolic profile. Further studies with inclusions of more than 5.0 g/kg should be carried out to confirm the inhibitory

effect of *Tribulus terrestris* on protozoa, followed by production performance experiments.

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