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DIGESTIBILITY, RUMEN FERMENTATION, RUMEN MICROBIOTA, AND LIPID PEROXIDATION IN SHEEP SUPPLEMENTED WITH LIPOIC ACID

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ABSTRACT

Lipoic acid (LA) reduces oxidative stress and stimulates the immune system. However, data on its use in ruminants is limited. The objective of this study was to determine the effects of LA supplementation on free radical production, rumen variables, and feed digestibility in sheep fed high-grain diets. Nine rumen-cannulated Suffolk sheep were randomly assigned to three treatments: control (LA_0), no LA inclusion; supplementation of 10 (LA_{10}) mg LA kg^{-1} body weight (BW); and supplementation of 20 (LA_{20}) mg LA kg^{-1} BW. *In situ* (ISDMD) and *in vitro* (IVDMD) degradation of dry matter and ruminal variables were evaluated. Total bacteria (TB), cellulolytic bacteria (CB), and protozoa (P) were also determined. Thiobarbituric acid reactive substances (TBARS) were evaluated in blood samples at the beginning and end of each experimental period. The ruminal variables and IVADDM were analyzed using a PROC GLM model with a repeated 3 × 3 latin square design, while means were compared using pairwise Tukey tests. LA supplementation did not affect ($p > 0.05$) the ruminal variables under study. LA modified ($p < 0.05$) ISDMD at 4 h in LA_{10}, while no differences were found in terms of IVDMD among the treatments. The LA dose of 10 mg kg^{-1} BW increased CB ($p < 0.05$) and decreased TB ($p < 0.05$). TBARS differed ($p < 0.01$) among the treatments and evaluation periods and were higher ($p < 0.05$) 15 d after administration. In conclusion, LA supplementation in sheep had an antioxidant effect, reducing oxidative stress 15 d after administration, with slight differences in the ruminal variables evaluated.

Key words: antioxidant, *in situ* digestibility, oxidative stress, peroxidation.

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INTRODUCTION

In ruminants, oxidative stress may be involved in several pathological conditions and damage of macromolecules, which are relevant to animal production and general animal welfare (Celi and Gabai, 2015), while it is positively related to high grain or high-concentrate diets of ruminant (Ma et al., 2021). Extreme quantities of reactive oxygen species (ROS), exceeding the body antioxidant capacity, cause oxidative stress (Cecchini et al., 2018). Free radicals, ROS and reactive nitrogen species are highly active chemical compounds formed continuously in plants and animals as a natural by-product of aerobic metabolism or in response to stress (Anthony et al., 2021). In particular, lipid peroxidation involves the oxidation of polyunsaturated fatty acids due to the presence of several double bonds in their structure, resulting in the production of peroxides, ROS, and other reactive organic free radicals (Nita and Grzybowski, 2016). Thiobarbituric acid reactive substance (TBARS) assay is a method to detect lipid peroxidation, which has been widely used as a good indicator of the levels of oxidative stress within a biological fluid (De León and Borges, 2020).

Lipoic acid (LA), a dithiol compound derived from octanoic acid, is a short-chain fatty acid that functions as a cofactor in vital energy-producing reactions and thus plays a major role in energy metabolism (Li et al., 2014). Because of its unique sulfur-containing structure, it can scavenge several types of free radicals, including ROS (El Barky et al., 2017). Studies on animals have shown that an increase in antioxidant levels in organisms can reduce cytokine production (Yan et al., 2014). In this regard, antioxidant defense systems may involve endogenous antioxidant enzymes or exogenous antioxidants from the diet, such as vitamin E, vitamin C, and β-carotene, which protect cells from ROS (Karakus et al., 2009). Some studies in broiler chickens have reported that LA supplementation (500 mg kg^{-1} feed) can increase total glutathione levels and improve hepatic total antioxidant capacity and antioxidant activity (Guo et al., 2014; Wasti et al., 2021). Currently, animals grown for meat are fed large amounts of grains to encourage weight gain and reduce the fattening period. However, diets with high levels of grain increase oxidative stress due to their lower amount of antioxidant vitamins compared to forage-based diets (Mercier et al., 2004). As a result of increased oxidative stress, immune defense mechanisms tend to decrease. To date, there is no information on the role of LA supplementation on sheep. Therefore, the objective of this research was to determine the effects of LA supplementation on free radical production, rumen variables, and feed digestibility in sheep fed high-grain diets.

MATERIALS AND METHODS

Animals, treatments, and diets

The Animal Welfare Committee of the Postgraduate College, Campus Montecillo approved the experimental procedures according to regulations established by Animal Protection Law promulgated by the State of Mexico. Nine Suffolk sheep (55 ± 10 kg live weight, 2 - 2.5 years-old) provided with a ruminal cannula were housed in individual metabolic cages (0.62 x 0.80 m) and randomly assigned to treatments as part of a repeated 3 × 3 Latin square design. Animals were offered water and feed *ad libitum* to meet their nutritional requirements (NRC, 2007). Feed was offered twice a day (08:30 and 16:30 h). The sheep were supplemented orally with different doses of LA adjusted to animal weight 10 days before offering the morning diet. LA was deposited in white tissue paper impregnated with molasses to facilitate oral administration, with the help of a 10 mL syringe cut in the upper part where the prepared supplement was placed. The treatments included in the experimental design were as follows: control, without LA supplementation (LA_0); administration of doses of 10 (LA_{10}) and 20 (LA_{20}) mg LA kg^{-1} body weight (BW). Three periods of 23 days were evaluated (including 15 days of adaptation to the diet and 8 days of sampling) with an interval of 30 days between periods in order to avoid residual effects between samples due to the administration of LA. The diet included concentrate (Table 1) and forage in a ratio of 85:15; the forage offered was chopped oat hay. Dry matter (method number 934.01), crude protein (method number 992.15), and ash were determined in the experimental diet (method number 945.18) (AOAC, 2005); acid detergent fiber and neutral detergent fiber were determined according to Van Soest et al. (1991).

In vitro anaerobic degradation of dry matter

In vitro anaerobic dry matter degradation (IVADMD) at 72 h was determined to evaluate the effect of LA on the activity of anaerobic bacteria (Varel et al., 1989) using glucose, cellobiose, starch and ruminal fluid (GCS-RF) as a culture medium containing 100 ml, 52.6 ml of destiled water, 30 mL of RF clarified, 5.0 mL of mineral solution I [6 g K_2HPO_4 in 1000 mL of destiled water], 5.0 mL of mineral solution II [6 g KH_2PO_4 + 6 g $(NH_4)_2SO_4$+12 g NaCl+2.45 g MgSO4+1.6 g $CaCl_2{\cdot}H_2O$ in 1000 mL of destiled water], 5.0 mL of 8 % solution of Na2CO3, 0.2 g

Table 1. Formulation and chemical composition of the diets provided to the sheep.

Item	%	Chemical composition	
Ground maize	86	Dry matter (%)	13.23
Maize straw	6	*As % dry matter*	
Molasses	3.7	Crude protein	12.11
Urea	1.36	Acid detergent fiber	9.55
Soybean meal	1.8	Neutral detergent fiber	30.72
Minerals mix[1]	0.05	Ash	3.56
Vitamin ADE[2]	0.6		

[1]P, 6.0%; Ca, 16%; Na, 10%; K, 0.2%; Zn, 0.3%; Cu, 0.06%; Fe, 0.18%; S 0.4%; Mg, 0.2%; Mn, 0.2%; I, 20 ppm; Co, 6 ppm; Se, 12 ppm. [2]Vitamin A, 50000 UI kg^{-1}; Vitamin D, 10000 UI kg^{-1}; Vitamin E, 250 UI kg^{-1}.

of trypticase peptone, 0.1 mL of 0.1 % solution of resazurine, 0.1 g of yeast extract, 2 mL of sulfide cysteine solution [2.5 g L-cysteine in 15 mL of 2N NaOH+2.5 g Na_2S-9H_2O gauged to 100 ml with H_2O, bubbled with CO_2 for 10 min and sterilized], 0.06 g of D-(+)-glucose, 0.6 g cellobiose and 0.06 g starch; and concentrations of 0.055 and 0.110 mg for LA_{10} and LA_{20}, respectively, calculated based on ruminal fluid volume in 50 kg BW in sheep and a maximum LA supplementation dose of 500 mg according to the experimental treatments. IVADMD was calculated by the following equation:

$$\% \text{ degradation} = \frac{(\text{final weight-paper weight})}{(\text{sample weight})}$$

Ruminal variables

On the last two days of each experimental period, 150 mL of ruminal liquid was collected from the ventral part of rumen, at 0 and 3 h after the morning feeding, directly through the ruminal cannula in a precipitate glass. The samples were filtered with a gauze and pH was immediately measured with a portable pH meter (model HANNA HI-255 Combined Meter, Germany). The ruminal liquid samples were stored in 10 mL tubes and stabilized with metaphosphoric acid (25%) in a 4:1 ratio and frozen at -4° C until analysis. The ammonia nitrogen (NH_3-N) concentration (McCullough, 1967) was determined through absorbance in an ultraviolet light spectrophotometer (Varian™, model Cary-1-E) at 630 nm. Volatile fatty acids (VFA) were determined according to Erwin et al. (1961) using a chromatograph (Perkin Elmer™ Claurus 500) with a FFAP Elite capillary column.

***In situ* ruminal degradation of dry matter**

In situ ruminal degradation of dry matter in the diets were estimated using the nylon bag technique. Five grams of ground feed (Thomas-Wiley mill, model 4, sieve 2 mm) was placed in a polyester bag (15 × 15 cm) with a 50 µm diameter pore size. The bags were incubated in the rumen for 4, 12, 24, and 48 h in reverse order of incubation time and extracted at the same time. Duplicate bags were incubated during each sampling period following the methodology described by Mehrez and Orskov (1977). After the incubation periods, the bags were immersed in water containing ice cubes to stop fermentation and immediately washed manually with water at room temperature until the water ran clear. After washing, the bags were dried for 48 h at 60 °C in a forced-air oven (FE-291 AD, FELISA™, Mexico), transferred to a desiccator for 30 min, and then weighed. The incubation residues were ground in a mill with a 1 mm sieve and used to determine DM (AOAC, 2005).

Total bacteria, cellulolytic bacteria, and protozoa

The concentration of total bacteria (TB) and cellulolytic bacteria (CB) was determined by the most probable number method (Harrigan and McCance, 1990). The medium was prepared in 10 mL tubes, using a volume of 4.5 mL of anaerobic culture medium based on GCS-RF (Cobos and Yokohama, 1995), incubated at 38°C for 72 h, and then inoculated with 0.5 mL of the rumen fluid collected from the sheep to obtain three different dilutions of the three treatments. The dilutions used to determine the concentration of total bacteria (TB) and cellulolytic bacteria (CB) were 10^{-1} to 10^{-14} and 10^{-1} to 10^{-10} with three replicates, respectively. For CB quantification, a Whatman 541 paper strip (0.5 x 2.0 cm) was added for CB quantification. After inoculation, the tubes were incubated at 38°C for 24 h for TB and 10 d for CB. Positive growth was confirmed by the degradation of the Whatman 51 paper. Confidence intervals were established at 95 % according to Harrigan and McCance (1990). Total protozoa (TP) in the rumen fluid was calculated by the following equation:

Protozoa mL^{-1} = ruminal liquid (mean reading: 2.5) x 10^4 (Ley de Coss et al., 2011); the count was made at 3 h of incubation.

Plasma peroxidation

To determine TBARS, a volume of 5 mL of blood was collected from the jugular vein with vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The serum was obtained by centrifugation at 823 *g* for 10 min. The samples were taken during each experimental period on three consecutive days (days 1, 2, and 3 in the first period; days 15, 16, and 17 in the second period; days 21, 22, and 23 in the third period), a composite sample was obtained with three replicates each, per experimental period. The samples were kept refrigerated (4 ºC) until arrival at the laboratory and processed on the same day using the technique described by Zentella et al. (1993).

Statistical design and analysis

Statistical Analysis System (SAS, 2004) was used for data analysis. The ruminal variables and IVADDM were analyzed using a PROC GLM model with a repeated 3 × 3 Latin square design. Means were compared with pairwise Tukey tests. *In situ* degradation variables were analyzed with a similar design using a PROC MIXED model, including the effects of the treatment and time, and the interaction of treatment × time. The results were expressed as least square means ± standard errors and were compared with Tukey tests. Ruminal DM degradation was fitted to an exponential PROC NLIN model according to Orskov and McDonald (1979) using the following equation:

$$Y = a + b\,(1 - e^{-ct})$$

where Y = dry matter degradation (%), a = rapidly soluble fraction, b = degradable fraction, $a + b$ = potential degradability (%), c = fractional rate of degradation (h^{-1}), and t = incubation time (h). The undegradable fraction was estimated as a proportion as follows: 100 – (a + b).

RESULTS AND DISCUSSION

***In vitro* anaerobic degradation of dry matter**

In vitro degradability of dry matter at 72 h did not differ among the treatments ($p > 0.05$) (Table 2). Kour et al. (2014) reported that there were no negative effects on the *in vitro* digestibility of dry matter when adding Kinnow mandarin residues with antioxidant properties to mixtures of isonitrogenous concentrates. In contrast, a recent study conducted by Wu et al. (2022) reports in vitro dry matter degradation ranging from 73 to 77% by including 20, 40 and 60 g kg^{-1} cysteamine, which neutralizes oxidative stress in animals, in forage-concentrate substrate (30:70). In ruminal fermentation, a high forage substrate generally leads to an increase in the number of fiber-degrading microorganisms, while a low forage content results in an increase in the number of starch-degrading microorganisms. It is possible that the dose of lipoic acid added to the substrates was not sufficient to develop a positive effect on

Table 2. *In vitro* degradability of dry matter, rumen pH, NH_3-N and volatile fatty acid concentration in sheep supplemented with lipoic acid at 0 and 3 hours after feeding.

Item	Treatment				
	LA_0	LA_{10}	LA_{20}	SEM	*p-value*
In vitro degradability dry matter at 72 hours	49.01	47.10	48.62	1.11	0.51
pH0 hours/pH3 hours	6.07/6.01	6.40/6.04	6.12/5.82	0.12/0.14	0.28/0.71
NH_3-N (mg dL^{-1})3 hours	12.31	11.85	6.34	4.51	0.37
Total volatile fatty acid (mmol L^{-1}) 3 hours					
Acetate	59.98	60.10	61.57	3.76	0.51
Propionate	38.35	35.32	31.87	5.16	0.90
Butyrate	7.77	7.96	9.52	1.03	0.65
Volatile fatty acid (Molar %) 3 hours					
Acetate	56.96	59.86	60.31	3.03	0.76
Propionate	34.75	32.17	30.08	3.69	0.89
Butyrate	8.27	7.95	9.61	1.20	0.62
Acetate:Propionate (A:P)	1.64	1.86	2.00	0.39	0.88

[a,b] Means in the same line with different letters are statistically significantly ($p < 0.05$).

LA_0: control, no lipoic acid supplementation; LA_{10}: supplementation of 10 mg lipoic acid kg^{-1} BW; LA_{20}: supplementation of 20 mg lipoic acid kg^{-1} BW. SEM = standard error of mean.

rumen microorganisms. To date, no studies have reported on the effects of lipoic acid on the rumen microbiome.

Ruminal variables

The pH at 0 and 3 h after feeding (Table 2) did not different among the treatments ($p > 0.05$). However, pH homogeneously decreased at 3 h in all treatments, showing no significant differences ($p > 0.05$). The ruminal pH values in sheep fed a diet of 85% feed concentrate and 15% forage supplemented with or without LA remained within a range of 5.82 to 6.04 after 3 h of providing the diet (Table 2). Rumen pH is best measured at its lowest value (i.e., 2-4 hours after feeding a concentrate meal or 4-8 h after offering a fresh total mixed ration) (Grünberg and Constable, 2009), while optimum diet fermentation and fiber digestion are achieved at a ruminal pH between 6.0 and 6.4 (Antanaitis et al., 2020). Most bacteria grow well at a pH between 6.2 and 7.0. Nevertheless, cellulolytic species such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens,* and *Ruminococcus albus* decrease their growth as pH is closer to 5.5, which implies a minimal bicarbonate concentration in the medium (Shi et al., 1997). De Brito et al. (2007) reported that ruminal pH can oscillate between 5.5 and 7.5 depending on the diet and that a pH value of 6.46 is considered optimal for microbial synthesis.

Supplementation with LA had no effect ($p > 0.05$) on NH_3-N concentrations in ruminal liquid (Table 2). Dietary protein is the main source of ruminal NH_3-N, and its concentration is affected by rates of ruminal nitrogen degradation and uptake because of the nitrogen rate used by rumen microorganisms (Cui et al., 2021). The mean concentration of NH_3-N varied from 6.34 to 12.31 mg dL^{-1}, which is sufficient to promote bacterial growth, including cellulolytic bacteria (Hoover, 1986; Da Silva et al., 2014).

VFA concentration in the rumen of LA-supplemented animals was similar ($p > 0.05$) to the control (Table 2), which is consistent with the findings of Robles-Rodríguez et al. (2022) who added 0, 40, 80 and 120 ppm LA in the diet of sheep. In the present study, the VFA concentration in the rumen is within normal limits (70 to 150 mM) to support microbial growth (McDonalds et al., 2012). Butyrate concentration increased in the ruminal liquid at pH 5.8, while a decrease was observed at pH 6.04, which is in agreement with Miguel et al. (2019). This can be explained by the results of Louis and Flint (2009), who demonstrated that two large butyrate-producing bacterial groups, the *Roseburia* species, *Eubacterium rectale* and *Faecalibacterium prausnitzii*, thrived at pH 5.5, while their population decreased at pH 6.5. The supplementation with 10 and 20 mg kg^{-1} of LA in the diet had no significant impact on ruminal variables in our study. These results can be attributed to the fact that LA structure may change during fermentation and metabolism in ruminants, and thus structure changes may cause changes in antioxidative status (Wang et al., 2017). Lipoate (the deprotonated charge form of lipoic acid that dominates at pH of above 4.7) metabolism can be found in most bacterial, fungal, and protozoan organisms (Spalding and Prigge, 2010). However, further research is required to determine the role of LA and its effects on the ruminal microbiome as well as its action mechanism under different feeding systems (Robles-Rodríguez et al. 2022).

***In situ* ruminal degradation of dry matter**

In situ degradation of the diet offered to the sheep differed ($p < 0.05$) between treatments after 4 and 12 h of incubation (Fig. 1). Differences were observed among the treatments during the first four hours. The highest *in situ* DM degradation occurred with LA_{20} (19.4±2.1) followed by LA_{10} (15.3±1.2) and the LA_0 (9.3±2.5). Similarly, LA_{20} (28.5±1.9) and LA_{10} (25.5±3.1) showed greater degradation ($p < 0.05$) than the control (21.0±1.6) after 12 h of incubation.

It is important to note that differences in *in situ* DM degradation between the LA treatments could be explained by the effect of the diet and not by LA degradation, attributed to the fact that LA structure may change during fermentation and metabolism in ruminants (Wang et al., 2017). However, as the animals consumed a diet high in grains and easily fermented carbohydrates, high *in situ* DM degradation was observed at different incubation times. The effect detected during the first four hours showed that, DM degradation at the highest dose of LA was significantly higher than in the other two treatments. Vázquez-Añón and Jenkins (2007) observed that synthetic antioxidants (ETQ: ethoxyquin, TBHQ: butyl hydroxyquinone) in ruminants improve fiber digestibility in the rumen as well as fat content and milk production during late lactation. Regarding supplementation of antioxidants in dairy cow diets, several studies have reported positive effects on some ruminal parameters, including lower ammonium nitrogen production, VFA, and pH and improved fiber digestibility (Hassan et al., 2020). Therefore, supplementation with antioxidants appears to favor changes in microbial metabolism as well as improve cellulolytic activity and the use of nitrogen in the diet, increasing rumen microbial protein synthesis (Vázquez-Añón and Jenkins, 2007; Tian et al., 2023).

Table 3 shows the results obtained for *in situ* DM degradation of the diets.

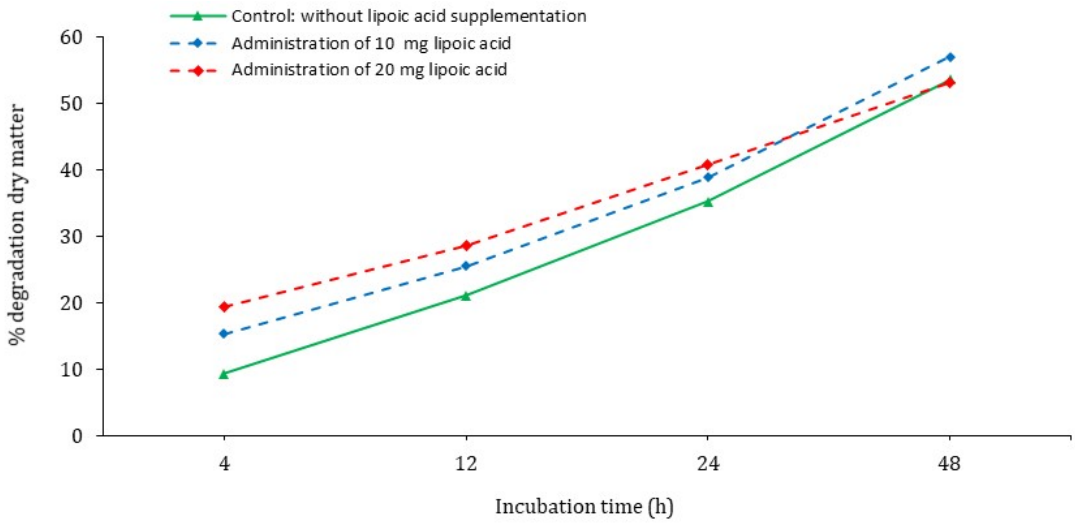

Fig. 1. Variation of *in situ* dry matter degradation of diets with or without the inclusion of lipoic acid.

Table 3. *In situ* dry matter degradation of the diet with or without the inclusion of lipoic acid.

	a	*b*	*c*	*(100 – a + b)*	*a + b*
	Mean ± SE	**Mean ± SE**	**Mean ± SE**		
LA_0	1.04 ± 2.22	92.79 ± 25.90	1.96 ± 0.008	6.17	93.83
LA_{10}	1.41 ± 1.77	75.07 ± 10.25	2.70 ± 0.007	23.52	76.48
LA_{20}	0.88 ± 1.82	58.64 ± 4.33	4.64 ± 0.008	40.48	59.52

a = Soluble fraction, *b* = Potential degradability of insoluble fraction, *c* = Rate of the potentially degradable fraction, (100 – *a* + *b*) = non-digestible fraction, *a* + *b*= Potential degradability of the substrate at 48 h of rumen incubation, LA_0: control, no lipoic acid supplementation; LA_{10}: supplementation of 10 mg lipoic acid kg^{-1} BW; LA_{20}: supplementation of 20 mg lipoic acid kg^{-1} BW. SE = standard error.

Total bacteria, cellulolytic bacteria and protozoa

The total concentration of bacteria in the ruminal liquid of sheep supplemented with LA_{10} (0.20×10^{11} mL^{-1}) decreased ($p < 0.05$) compared to LA_0 (7.5×10^{11} mL^{-1}) and LA_{20} (20×10^{11} mL^{-1}) (Table 4). On the other hand, the concentration of cellulolytic bacteria in the ruminal liquid of sheep supplemented with both doses of LA (LA_{10}, 150×10^5 mL^{-1}; LA_{20}, 1.5×10^5 mL^{-1}, respectively) increased ($p < 0.05$) compared to the control (0.75×10^5 mL^{-1}). In addition, no differences were observed in the concentration of protozoa (Table 4).

The presence of bacteria, including cellulolytic bacteria and protozoa in the rumen, changes according to the rations and dietary ingredients consumed by the host since these organisms prefer specific substrates. Notably, the ruminal environment is a complex system that is influenced by diet, but also by factors such as feed intake, passage rate, and the existing microbial population and their interactions, which then determine rumen fermentation indicators (ruminal pH, NH_3-N and VFA) (Castillo-González et al., 2014). In the present study, the concentration of total bacteria differed in the LA_{20} treatment but remained within the normal range (10^{10} to 10^{11} mL^{-1}) observed in sheep fed a high-grain diet.

The increase in the concentration of cellulolytic bacteria in the treatment LA_0 is related to the higher production of acetic acid than propionic acid. Miura et al. (1980) mentioned that the use of pyruvic acid is more efficient than acetic acid because it acts via the acetyl-CoA pathway. This may also occur following the addition of LA, because this vitamin intervenes as a cofactor in energy metabolism (Lodge and Packer, 2000).

Although the rumen is an anaerobic medium, ruminal bacteria can be exposed to oxygen

Table 4. Concentration of total bacteria, cellulolytic bacteria, and protozoa in rumen liquid of sheep fed with or without lipoic acid supplementation.

	Treatment			
Concentration	**LA_0**	**LA_{10}**	**LA_{20}**	**SEM**
Total bacteria ($x10^{11}$ mL^{-1})	17.50 a	0.20 b	20 a	9.85
Confidence interval ($P < 0.05$)	1.6 – 35.1	0.43 – 0.95	4.27 – 93.6	
Cellulolytic bacteria ($x10^5$ mL^{-1})	0.75 a	1.05 ab	1.50 b	47.62
Confidence interval ($P < 0.05$)	0.16 – 3.51	32.05 – 702	1.49 – 32.8	
Protozoa ($x10^4$ mL^{-1})	98.03 a	142.14 a	136.60 a	31.65

[a,b] Means in the same line with different letters are statistically significantly ($p < 0.05$).
LA_0: control, no lipoic acid supplementation; LA_{10}: supplementation of 10 mg lipoic acid kg^{-1} BW; LA_{20}: supplementation of 20 mg lipoic acid kg^{-1} BW. SEM = standard error of mean.

toxicity. For this reason, these bacteria have enzymatic defense mechanisms. Exposure to oxygen can occur when the ruminal microbiota is transferred from mother to offspring or when large quantities of contaminated water are consumed (Mead and Jones, 1981). Holovská et al. (2002) conducted *in vitro* evaluations of the growth of bacteria such as *Streptococcus bovis* in media containing mercury chloride and in media with plants containing high levels of antioxidants, such as vitamin A. The authors also evaluated the effects of the antioxidant activity of enzymes such as SOD and GSHPx on ruminal bacteria exposed to environmental stress factors and AOS, and concluded that AOS increased the SOD action of *Streptococcus bovis*, favoring the growth of these bacteria and minimizing the environmental stress induced by mercury. There is additional evidence that antioxidant activity affects ruminal bacteria given that vitamins are essential co-factors of different enzymes (Madigan et al., 2003). Furthermore, Yu et al. (2020) indicates that dihydropyridine can increase the antioxidant capacity of dairy cows with favorable effects on ruminal microorganisms under heat stress.

In terms of protozoa concentrations, no differences were observed among the treatments, while concentrations of these organisms were within the range of values previously reported for healthy animals (Ley de Coss et al., 2011). However, protozoa have been found to intervene in diverse functions during the metabolization of nutrients in ruminants (Abubakr et al., 2013), mainly during the metabolization of lipids. Some authors have reported that protozoan populations increase in animals fed diets high in grains such as barley, maize, and sorghum (Franzolin and Dehority, 1996; Towne et al., 1990). Thus, protozoa act as a stabilizing agent of starch fermentation in the rumen of grain-fed ruminants by engulfing starch granules (Willians and Coleman, 1997; Newbold et al., 2015). Meanwhile, Hristov et al. (2001) reported that the concentration of protozoa (470×10^3 mL^{-1}) was 42 % lower in ruminants fed a diet high in barley grain than in ruminants fed an intermediate level (840×10^3 mL^{-1}). A study conducted by Veira et al. (1983) showed that the concentration of ruminal protozoa varies due to the effect of type of animal, diet, and level of intake. On the other hand, Park and Yu (2018) reported that ascorbic acid, glutathione and α-ketoglutarate enhanced the reactivation of cryopreserved stock cultures of rumen protozoa (*Entodinium caudatum* and *Epidinium caudatum*). However, there is little information on the effect of LA on the rumen microbiome and its physiology, which requires further investigation.

Plasma peroxidation

Finally, TBARS differed ($p < 0.01$) among the treatments and between sampling periods, showing an antioxidant effect after 15 days of supplementation in LA_{10} (Table 5). TBARS was assessed in plasma as an indicator of lipid peroxidation and the antioxidant action of LA supplementation. Doses of 10 and 20 mg LA kg^{-1} day^{-1} in sheep diminished lipid peroxidation after 15 d of supplementation, which can be attributed to a reduction in oxidative stress. This result is consistent with Wang et al. (2017), who indicated that LA resulted in reduced oxidative stress by improving the antioxidant capacity in Hainan black goats. Similarly, Yang et al. (2023) included doses of 0, 300, 450, 600 and 750 mg kg^{-1} LA in the diet for Duhu sheep and reported an increase in the activity of antioxidant enzymes, inhibiting muscle oxidation through the improvement of the antioxidant capacity at doses of 600 mg kg^{-1} in Tan lamb diets (Luo et al., 2022). In addition, El-Senousey et al. (2018)

Table 5. Thiobarbituric acid reactive substances concentration (TBARS: nmoles mg^{-1} protein) in blood serum of sheep fed with or without lipoic acid supplementation.

Sampling days	Treatment			SEM	*p-value*
	LA0	LA_{10}	LA_{20}		
1	0.051 a	0.042 a	0.029 b	0.021	0.0025
15	0.041 a	0.024 b	0.020 c	0.018	0.0085
21	0.031 a	0.034 a	0.019 c	0.035	0.0001

[a,b] Means in the same line with different letters are statistically significantly ($p < 0.05$).
LA_0: control, no lipoic acid supplementation; LA_{10}: supplementation of 10 mg lipoic acid kg^{-1} BW; LA_{20}: supplementation of 20 mg lipoic acid kg^{-1} BW. SEM = standard error of mean.

reported that LA (500 mg kg^{-1} diet) is more effective in reducing oxidative stress in broilers than vitamin C or E, which can be attributed to the antioxidant capacity of LA and its ability to regenerate vitamins C and E. Furthermore, Wasti et al. (2021) reported that the supplementation of LA (500 mg kg^{-1} diet) significantly improved antioxidant capacity and heat shock and can be considered one of the potential strategies to mitigate heat stress in broilers. Williams et al. (2002) supplemented LA in a horse diet at a dose regimen of 10 mg kg^{-1} day^{-1} for 14 d and observed a moderate reduction in oxidative stress following minimal physical activity. In this case, lipid peroxidation was measured by evaluating total glutathione and glutathione peroxidase in blood plasma. The reduced form of lipoic acid (dihydrolipoic acid) is able to reduce oxidized forms of other cellular antioxidants, including glutathione, α-tocopherol, ascorbic acid, as well as quenching ROS and RNS (Ponnampalam et al., 2022). Likewise, Nanji and Zenad (2015) conducted a study on LA as a therapeutic antioxidant agent in sheep in order to determine the effects of oxidative stress induced by cadmium toxicity, and concluded that LA has an ameliorating effect on cadmium toxicity as an antioxidant substance.

CONCLUSIONS

In the present study, supplementation with lipoic acid at a dose of 10 mg kg^{-1} BW in sheep fed high-grain diets increased the cellulolytic bacterial population in the rumen without modifying other ruminal variables. In addition, supplementation with lipoic acid improved the antioxidant capacity of the diet, as confirmed by the reduction in lipid peroxidation products after the administration of lipoic acid for 15 d.

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