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Use of grape residue flour in lactating dairy sheep in heat stress: Effects on health, milk production and quality

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ABSTRACT

The objective of this study was to evaluate the effects of grape residue flour (GRF) on antioxidant activities, biochemistry variables, components of the immune system and milk production and quality of Lacaune sheep in heat stress. Twenty-seven multiparous lactating sheep [50 ± 1.8 days (d) milking] were stratified by initial body weight, age, date of lambing and milk production and assigned randomly to 1 of 3 treatments (9 sheep/treatment): no GRF supplementation (control group) or supplementation at 1% (10 g/kg GRF) or 2% (20 g/kg GRF) of GRF (bark and seed) in the concentrate (grains and minerals mixture). Each ewe received 0.8 kg/d of concentrate, 3.6 kg/d of corn silage, and 0.25 kg/d of *Cynodon* spp hay. Milk production along with blood and milk samples were collected on d 1, 10 and 15. The 2% GRF sheep had increased serum concentrations of superoxide dismutase and glutathione peroxidase activity on d 15 compared to control sheep. Over time (d 10 to 15), lipid peroxidation was reduced in 2% GRF sheep. Total serum antioxidant capacity was greater in 2% GRF sheep compared to control sheep on d 10 and 15. Superoxide dismutase and glutathione peroxidase activity in milk samples were greater in 2% GRF sheep compared to control sheep. Supplementation with GRF did not affect milk production but GRF sheep were more efficient compared to control sheep. Protein and lactose concentrations were similar between treatments, but total solids and fat concentrations were greater in 2% GRF sheep compared to control sheep on d 15. Somatic cell count was reduced in GRF sheep compared to control sheep. In summary, supplementation with 2% GRF in dairy sheep in heat stress resulted in antioxidant and anti-inflammatory responses, which improved milk quality and reduced somatic cell count and lipid peroxidation.

1. Introduction

In dairy sheep, energy requirements increase with gestation, lambing, and lactation, which leads to an increase in cellular respiration and consequently free radical production and oxidative stress (Mutinati et al., 2013). This occurs due to an imbalance between production of free radicals or reactive metabolites (oxidants) and antioxidants. When antioxidants do not rapidly neutralize free radicals, it leads to the accumulation of oxidants in tissue and consequently damages biomolecules and organs (Duracková, 2009). This effect may be greater when the animal is under heat stress. According to the literature, exposure of sheep to elevated ambient temperature negatively affects biological functions which is reflected in the impairment of production and reproductive traits. Elevated ambient temperatures increase the dissipation of excess body heat in order to negate the excessive heat load. Further, heat stress causes a decrease in feed efficiency and utilization, causes a disturbance in water, protein, energy and mineral balances, and affects enzymatic reactions, hormonal secretions and blood metabolites (Marai et al., 2007).

To maintain the equilibrium between production and elimination of free radicals, the organism utilizes 3 methods of protection: 1) enzymes interfere with free radical formation, 2) antioxidants neutralize

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molecules with oxidizing action, and 3) repairing the system, by recognizing compromised molecules and removing them (Duracková, 1998). Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase are enzymes with antioxidant action (Duracková, 2009). Further, exogenous substances such as tocopherol (vitamin E) and flavonoids also exhibit antioxidant potential (Duracková, 2009; Silva et al., 2010).

Antioxidants compounds are capable of donating electrons and neutralizing free radicals, resulting in the prevention of cellular lesions (Saeidnia and Abdollahi, 2013). Due to the numerous benefits, the search for natural antioxidants in food products, cosmetics and pharmaceuticals has been a primary objective in the last 20 years (Laguerre et al., 2007). Feeds with nutraceutical properties have been sought after by researchers, as recommended by nutritionists, due to their function in the prevention and treatment of disease (Moraes and Colla, 2006). Historically, antioxidants were added to food to avoid lipid peroxidation and rancidification during processing and storage (Salami et al., 2016). According to Salami et al. (2016), antioxidants present in the diet combat oxidative stress in livestock animals and in doing so improves health, welfare, nutritional and organoleptic quality, as well as shelf life of animal products. Resveratrol is a phenolic compound found in grapes (Abe et al., 2007) which possesses antioxidant, antimicrobial, and anti-inflammatory properties (Karami et al., 2018).

Previous research has shown that resveratrol plays a role in the prevention of apolipoprotein-B peroxidation and is associated with low-density lipoproteins (LDL), which ultimately restores glutathione in plasma and tissue (Sahin et al., 2010). Quercetin is a flavonoid found in grapes in the glycosylated form and exhibits antioxidant properties (Behling et al., 2004). Flavonoids are considered effective antioxidants due to their ability to scavenge free radicals and by chelating metal ions (Kandaswami and Middleton, 1994). Antioxidant properties are directly due to the hydroxyl radical ($^{\cdot}OH$) and the superoxide anion ($O2^{-\cdot}$), both of which are highly reactive species involved in the initiation of lipid peroxidation (Behling et al., 2004).

The southern region of Brazil is known for its wine production, producing more than 500 tons of grapes in 2016 (IBGE, 2016). Increased wine production (and other grape derivatives) results in a large volume of waste which is currently not being utilized efficiently. Brenes et al. (2008) reported that the inclusion of grape residues in the diet has positive effects on the welfare of broilers and increased the amount of antioxidants present in the muscle. Similarly, Ebrahimzadeh et al. (2018) reported that the inclusion of grape residue in the diet of broiler chickens improved the immunological and antioxidant responses. In another recent study, Hamza and El-shenawyb (2017) supplied oral resveratrol to mice exposed to nicotine and observed reduced lipoperoxidation and increased antioxidant enzymes. In dairy cows, the inclusion of grape silage in the diet had positive effects on milk production due to the increase in antioxidant capacity in the group with the greatest addition of grape residue (Santos et al., 2014). Thus, our hypothesis is that inclusion of grape residue flour (GRF) in the diet, will lessen the negative effects associated with heat stress and ultimately improve the quality of milk and animal health.

The GRF is composed of catequin, epicatechin, quercetin, caempferol, and resveratrol; all of which are substances known to improve the antioxidant system (Abe et al., 2007). However, the biggest challenge with providing grape residue in animal feed is its rapid deterioration in natural form. Therefore, the grape residue will be processed into a flour which will then be added to the feed. Our hypothesis is that supplementation with this residue will minimize oxidative stress. Thus, the objective of this study was to evaluate the effects of grape residue flour on antioxidant activities, biochemistry variables, components of the immune system and milk production and quality of Lacaune sheep under heat stress.

2. Material and methods

2.1. Grape residue flour (GRF)

The GRF used was purchased from a natural products company (Essencial®). Chemical composition was analyzed according to AOAC (2000): dry matter (DM), method 930.15; crude protein (CP), method 976.05; ethereal extract (EE), method 920.39 and ashes, method 942.05. The concentration of neutral detergent fiber (NDFom) and acid (ADFom) was done according to the methodology of Van Soest et al. (1991) without sodium sulfite and amylase.

2.1.1. Resveratrol and quercetin content in GRF

One GRF sample (1 mg ml^{-1}) was analyzed by high performance liquid chromatography coupled with diode array and mass spectrometry detectors (HPLC-DAD-MS/MS). High performance liquid chromatography was determined by Shimadzu Prominence UFLC (Shimadzu, Kyoto, Japan) equipped with an Auto-Sampler (SIL-20AHT), two Shimadzu LC-20ADT reciprocating pumps connected to the degasser DGU20A3R, integrator CBM20A, UV–VIS detector DAD SPD-M20A, and oven CTO-20A. The HPLC system was coupled to the compact quadrupole time-of-flight (Q-TOF) mass analyzer (Bruker Daltonik GmbH, Bremen, Germany), which was controlled using Ot of Control Software. The parameters for analysis were set using negative ion modes with spectra acquired over a large range from 50 to 1200 m/z. Optimum values for ESI-MS were: capillary voltage of 4500V, drying gas temperature of 215 °C, drying gas flow of 10.0 L/min, nebulising gas pressure of 5.0 Bar, collision RF of 150 Vpp, transfer time of 70 s, and a pre-pulse storage of 5 ls. Automatic MS/MS experiments were performed using nitrogen as collision gas and by adjusting the collision energy values as follows: m/z 100, 20 eV; m/z 500, 30 eV; and m/z 1000, 35 eV. The MS data were analyzed using Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany). Analyses were carried out within the C-18 column (4.6 mm × 250 mm, Merck, Germany) packed with 5 µm diameter particles and within the C-18 pre-column (RP 18 5 µm, Merck, Germany). The first mobile phase (phase A) was two percent acetic acid at a pH of 4.2. The second mobile phase (phase B) used methanol, acetic acid, and distilled water at a ratio of 18:1:1, respectively. The gradient elution was 0 min: 20% of B, 0–25 min: 50% of B, 25 min: 20% of B, 30 min: 20% of B (end of run), at a flow rate of 0.8 ml/min. The peaks were identified by comparing the present results with the retention times and mass spectrums from the software library and external standards. The external standards included resveratrol, quercetin and rutin (all standards by Sigma-Aldrich, St Louis, MO, USA) between 1.5 and 24 µg/mL. Sample and standards were tested in triplicate and the results are presented as mean ± standard deviation (SD).

2.1.2. Determination of total phenolic compounds (TPC) and antioxidant activity by elimination of radicals by DPPH

For extraction, 0.5 g of GRF samples were dissolved in 50 ml of distilled water. The mixture was placed in an ultrasonic bath (70 W) for 3 h and remained in the dark for 3 more hours. The supernatant was filtered with quantitative filter paper ('Whatman' # 40) and stored in a 100 mL volumetric flask wrapped in aluminum foil. After extraction, extracts were stored in Eppendorf tubes and maintained at – 80 °C until analysis (Larrauri et al., 1997; Bertoletti et al., 2018).

Quantification of TPC was performed by Folin-Ciocalteu colorimetric method modified by Bonoli et al. (2004). An aliquot of each diluted sample was mixed with 0.5 ml of Folin-Ciocalteu reagent and was stirred for 1 min. Next, 2 ml of sodium carbonate (20%) was added in the mixture and stirred for 30 s. After 2 h of incubation, the absorbance was read at 750 nm in relation to blank. The standard curve was prepared by solutions of gallic acid in methanol. The concentration of TPC was obtained using an equation derived from the standard curve of gallic acid (expressed in mg of gallic acid equivalent per g of dry

sample; mg EGA/g). Data are presented as the mean ± SD of triplicates.

Evaluation of free radical scavenging activity in the extracts was determined by the antioxidant reduction capacity of DPPH radical according to Brand-williams et al. (1995). Five dilutions of each extract were prepared in test tubes and 0.3 ml of each diluted extract was added to 2.7 ml of DPPH radical (40 µg/ml). After incubation for 1 h in the dark, the absorbance was read at 515 nm relative to the blank prepared. Free radical scavenging activity was reported as the IC 50 (µg/mL), which is defined as the antioxidant concentration required to eliminate 50% of the DPPH present in the test solution. All tests were performed in triplicate and IC 50 values were reported as means ± SD of triplicates.

2.2. Animals and experimental design

The experiment was conducted at a commercial dairy sheep farm in Chapecó, Santa Catarina, Brazil. Twenty-seven multiparous lactating sheep [50 ± 1.8 days (d) milking] of the Lacaune breed, were stratified by body weight (70.6 ± 2.9 kg), age, date of lambing, and milk production and assigned randomly to 1 of 3 treatments (9 sheep/treatment): no GRF supplementation (control group; CON) or supply of 1% (1% of GRF) or 2% (2% of GRF) of GRF (bark and seed) in the concentrate (grains and minerals mixture). Each animal received 0.8 kg/d of concentrate, approximately 3.6 kg/d of corn silage and 0.25 kg/d of *Cynodon* spp hay divided into 2 daily feedings (07:00 h and 17:00 h; Table 1). In order to standardize feed intake, all sheep were fed individually, concentrate was offered first and after consumption (approx. 15 min) silage was offered. The sheep were housed in a covered feedlot with wood shavings on the floor and allocated to 3 pens (1 treatment/pen) located side by side. The experiment occurred over a15 day period with the first 10 days set as an adaptation period to the experimental diet, protocol similar to that described by Jaguezeski et al. (2018). Concentrate including GRF had less acceptance than control, but after 3 days of adaptation all sheep were consuming the total amount of concentrate offered.

The experiment was carried out in the south of Brazil, in a shed without air conditioning, with lateral openings. The experiment took place during the summer months and temperature was measured inside the building during the day. The minimum and maximum temperature recorded were 27 °C and 35 °C, respectively. The hottest temperature occurred around 1430 throughout the experimental period. The maximum and minimum temperature of each day during the experimental period are presented in Supplementary Fig. 1. These animals showed clinical signs of heat stress in the hottest times of the day, characterized by rapid breathing, intense salivation and open mouth, with exposure of the tongue of many animals.

Table 1
Ingredients and chemical composition of ingredients and experimental diets.

Ingredients	As fed (kg/day)			Dry matter (DM; kg/day)		
Corn silage (kg)	3.60			1.17		
Concentrate[a] (kg)	0.80			0.71		
Hay (kg)	0.25			0.23		
Chemical composition[b]	**Grape flour**	**Corn silage**	**Hay**	**Concentrate**		
				CON	**1% GRF**	**2% GRF**
DM, g/kg	934	326	892	889	890	892
Ash, g/kg DM	124	45.0	60.0	87.0	77.0	69.0
CP, g/kg DM	103	72.1	72.0	216	208	205
NDF, g/kg DM	333	401	644	78.0	78.0	85.0
ADF, g/kg DM	217	242	401	28.0	28.0	31.0
EE, g/kg DM	50.2	31.0	16.0	23.0	23.0	26.0

[a] Ingredients present in 100 kg of concentrate: ground corn (671 g/kg), soybean meal (277 g/kg), calcitic limestone (10 g/kg), sodium bicarbonate (4 g/kg) and 37 g/kg of premix (calcium mim. 180 max. 220 g; phosphorus min. 32 g; sodium min. 40 g; sulfur min. 20 g; magnesium min. 20 g; cobalt min. 16 mg; iodine min. 17 mg; manganese min. 420 mg; selenium min. 730 mg; zinc min. 730 mg; fluorine max. 600 mg; niacin min. 500 mg; vitamin A min. 95000 UI; vitamin D min. 20000 UI; vitamin E min. 350 UI; monensin sodium 1200 mg; *Sacharomyces cerevisae* 2,1 × 10 UFC).

[b] Note: DM (Dry matter), Ash (Ashes), CP (Crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber) and EE (ethereal extract).

2.3. Feed analysis

2.3.1. Chemical composition of concentrate, silage and hay

Feed samples were dried in a forced ventilation oven at 55 °C for 72 h, ground at 1 mm in a Willey mill, and analyzed for chemical composition: DM, CP, NDF, ADF and analyzed as described for GRF. It is important to note that α-amylase was used in the analysis of both concentrate and silage diets (Van Soest et al., 1991).

2.3.2. Total phenolic compounds (TPC) and antioxidant activity in the concentrate

The preparation and extraction of concentrate in the diet was the same as that used for GRF (section 2.1.2) and measurement of TFC and antioxidant activity (IC 50) followed the same methodology previously described for GRF analysis (section 2.1.2).

2.4. Milk measurement

Individual milk production was evaluated twice a day (0600 and 1700) on d 1, 10 and 15 using a meter (True Test®, Aukland, New Zeland) and daily milk production was obtained by determining the sum of both milking events. Productive efficiency (%) was calculated individually based on milk production on days 1, 10 and 15 of the experiment and the increase in milk production from day 1–10 and day 1–15 for each group. The difference in milk production was assigned a percentage which was then used in the statistical analysis for productive efficiency.

2.5. Blood and milk collection

One milk sample (40 mL) per animal was collected using the equipment WB HI/Pullout (Tru-Test®; which collects a homogeneous sample from each animal for the entire milking) on d 1, 10 and 15. Two mL of milk sample were transferred to microtubes and stored at −20 °C until further analysis of superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, total antioxidant capacity (ACAP), and lipid peroxidation (LPO) levels.

Blood samples were collected from the jugular vein in blood collection tubes without sodium heparin (for biochemical, oxidant and serum antioxidant analysis) and with EDTA (for haematological analysis) at 0700 before animals were fed. Immediately after collection blood samples were stored on ice. Blood samples without sodium heparin were centrifuged at 5100 g for 10 min. Serum was harvested and stored at −20 °C until further analysis. Blood samples containing EDTA were stored at 4 °C and haematological analysis was performed within 2 h after collection.

2.6. Milk analysis

2.6.1. Chemical composition

Concentration of fat, protein, lactose and total dry extract were determined using an infrared analyzer (LactoStar Funke Gerber®) and somatic cell count (SCC) was determined using a digital counter (Ekomilk Scan Somatic Cells Analyzer®).

2.6.2. Analysis of oxidants and antioxidants

Glutathione peroxidase activity was determined according to Wendel (1981) and results were expressed as U GPx/mg of protein. Activity of SOD was determined according to Beutler (1984) and results

were expressed as U SOD/mg of protein. The LPO concentrations were determined according to Monserrat et al. (2003) and results were presented as μmol CHP/ml of serum or milk. The ACAP concentrations were determined according to Amado et al. (2009) and results were presented as FU/mg of protein. Protein concentrations in serum were determined by the Coomassie blue method according to Read and Northcote (1981), using bovine serum albumin as a standard.

2.7. Blood analysis

2.7.1. Hematology

Erythrocyte, total leukocyte counts, and hemoglobin (Hb) content were measured using a semi-automated analyzer (Celm® 530). Hematocrit was obtained after capillary centrifugation (11.000 g for 5 min). Leukocyte differential counts were performed in blood smears stained with a commercial dye (Romanowsky method) and viewed with a light microscope at 1000x magnification (Feldman et al., 2000).

2.7.2. Serum biochemistry

Total protein (TP), albumin, urea, triglycerides and cholesterol were measured using a semi-automated analyzer (BioPlus 2000®) with commercial kits (Analisa®, Gold Analisa Diagnóstica, Belo Horizonte, Brazil). Globulin levels were obtained using the following formula: total protein – albumin.

2.7.3. Serum oxidant and antioxidant analysis

The methodology for analyses of LPO, ACAP, GPx and SOD were the same as described in milk analysis.

2.8. Feed intake

Feed intake was measured by weighing the amount of feed offered and orts on d 11 to 15.

2.9. Statistical analysis

Data were submitted to a Shapiro-Wilk normality test. Majority of the data were not normally distributed and were log transformed. Subsequently, statistical analysis was performed using a bilateral, two-way analysis of variance (ANOVA) for independent samples followed by Tukey's post hoc analysis. Tukey's post hoc analysis compared treatment groups (T0, T1 and T2) and time (days 0–10; days 0–10; and days 10–15). Values were considered significant at $P \leq 0.05$.

3. Results

3.1. Resveratrol, quercetin, total phenolic compounds (TPC) and antioxidant activity in GRF

Resveratrol and quercetin concentrations were 52.8 ± 9.4 and 23.4 ± 3.64 mg/g, respectively. The TPC concentrations were 87.4 ± 0.27 mg EAG/g and antioxidant activity against radical DPPH (IC 50) was 111.6 ± 3.45 μg/mL.

3.2. Total phenolic compounds (TPC) and antioxidant activity in diets

The TPC concentration increased with increasing GRF in the diet (Table 2). Similarly, the antioxidant activity (IC50) was greater in the 2% GRF compared to the 1% GRF diet (Table 2).

3.3. Milk production and composition

Milk production (L/sheep/day) did not differ ($P > 0.05$) between treatments throughout the study (Table 3). However, sheep in the GRF groups were more efficient ($P \leq 0.05$) compared to sheep in the CON group (d 1 to 10, and d 1 to 15; Table 3). Protein and lactose concentrations were not different ($P > 0.05$) between groups, but sheep in the 2% GRF group had greater ($P \leq 0.05$; Table 3) lactose concentrations on d 15 compared to d 1. Fat and total solids concentrations were greater ($P \leq 0.05$) in the 2% GRF group compared to CON group on d 15 (Table 3). The SCC was decreased ($P \leq 0.05$) in sheep on the GRF diets compared to the CON on d 15 (Table 3). Over time (d 1 to 15), milk production increased ($P \leq 0.05$) in all treatments as indicated by different letter superscripts between days in Table 3.

Table 2
Mean and standard deviation (…) of total phenolic compounds (TPC) and antioxidant activity (DPPH assay) of experimental diets.

Treatment/diets[a]	TPC (mg EAG/100 g of dry mater)	Antioxidant activity – DPPH assay - IC_{50} (μg/mL)
CON	0.04 (0.02)[c]	[b]
1% GRF	2.87 (0.90)[b]	712 (1.02)[a]
2% GRF	9.69 (0.80)[a]	422 (1.57)[b]

Note: When comparing total phenolic compounds (TPC) and IC_{50} levels between treatments, we verified a statistical difference between groups, illustrated by different letters in the same column ($P < 0.05$). Analyzes made in triplicate.

[a] No grape residue flour (GRF) supplementation (control group; CON) or supply of 1% (1% GRF) or 2% (2% GRF) of GRF in the concentrate.

[b] No antioxidant activity (IC_{50}) was detected in the control diet (T0), that is, there was no reaction with DPPH radical, visualized by color change.

3.4. Milk antioxidants and oxidants

Lipid peroxidation concentrations were reduced ($P \leq 0.05$) in 1% GRF and 2% GRF groups compared to CON on d 10 and 15 (Fig. 1A). Over time (d 10 to 15), LPO was reduced ($P \leq 0.05$) in the 2% GRF group. Total antioxidant capacity was greater ($P \leq 0.05$) in the 1% GRF and 2% GRF groups compared to CON on d 10 and 15 (Fig. 1B). Activity of SOD was increased ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15 but there was no difference between the 1% and 2% GRF groups (Fig. 1C). Activity of GPx was greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 15 (Fig. 1D). Total antioxidant capacity, SOD, and GPx activity increased from d 1 to 15 in the 2% GRF group.

3.5. Hematology

Hemoglobin, hematocrit and erythrocytes did not differ ($P \leq 0.05$) between treatment or time (Table 4). Total leukocyte concentrations were decreased ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and the 1% GRF and 2% GRF groups were decreased ($P \leq 0.05$) compared to CON on d 15 (Table 4). Lymphocyte and neutrophil concentrations ($P \leq 0.05$) were decreased in the 1% GRF and 2% GRF groups compared to CON on d 15 (Table 4). No differences were detected for the main effects of treatment and time ($P > 0.05$) for monocyte and eosinophil concentrations (Table 4). From d 1 to 15, total leukocyte counts were decreased ($P \leq 0.05$) in the 2% GRF group. Similarly, lymphocyte counts were also decreased ($P \leq 0.05$) in both the 1% and 2% GRF groups from d 1 to 15.

3.6. Serum biochemistry

Glucose concentrations were greater ($P \leq 0.05$) in the 2% GRF group compared to CON. Glucose concentrations differed from d 1 to 15 for all groups (Table 5). Cholesterol concentrations did not differ ($P > 0.05$) between groups or over time (Table 5). Triglycerides concentrations were greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15 and concentrations increased over time (d 1 to 15) in 2% GRF group (Table 5). Total protein and globulin concentrations were decreased ($P \leq 0.05$) in the 2% GRF group compared to CON on d

Table 3
Milk production and milk composition from sheep not supplemented with grape residue flour (GRF; control group; CON) or supplemented with of 1% (1% GRF) or 2% (2% GRF) of GRF in the concentrate.

Variable	Day	CON	1% GRF	2% GRF	P-values
Production (L)	1	1.42 ^{B} (0.31)	1.39 ^{B} (0.21)	1.37 ^{B} (0.32)	**0.54**
	10	1.50 ^{A} (0.34)	1.62 ^{A} (0.40)	1.63 ^{A} (0.33)	**0.50**
	15	1.51 ^{A} (0.36)	1.64 ^{A} (0.32)	1.65 ^{A} (0.29)	**0.39**
	p-values	**0.01**	**< 0.001**	**< 0.001**	
Productive efficiency (%)	1 to 10	8.54 (4.10) ^{b}	19.4 (5.91) ^{a}	25.7 (6.81) ^{a}	**< 0.001**
	1 to 15	7.86 (4.31) ^{b}	19.4 (5.43) ^{a}	25.0 (6.54) ^{a}	**< 0.001**
Chemical composition					
Protein (g/kg)	1	58.0 (1.10)	58.0 (1.20)	58.0 (2.00)	**0.95**
	10	57.0 (0.90)	59.0 (1.60)	58.0 (2.20)	**0.93**
	15	57.0 (0.80)	57.0 (1.80)	57.0 (1.50)	**0.96**
	p-values	**0.94**	**0.80**	**0.92**	
Fat (g/kg)	1	64.0 (5.90)	63.1 (4.76)	64.0 (5.41)	**0.90**
	10	63.1 (5.42)	59.6 (5.63)	67.6 (7.93)	**0.45**
	15	62.2 (3.82) ^{b}	65.4 (4.20) ^{ab}	69.8 (2.83) ^{a}	**0.03***
	p-values	**0.89**	**0.39**	**0.25**	
Lactose (g/kg)	1	55.0 (2.12)	56.4 (2.51)	55.6 ^{B} (2.60)	**0.93**
	10	57.3 (2.01)	54.6 (3.74)	56.0 ^{AB} (2.93)	**0.62**
	15	57.7 (1.98)	58.0 (2.23)	59.7 ^{A} (1.73)	**0.22**
	p-values	**0.96**	**0.18**	**0.05** $^{+}$	
Total solids (g/kg)	1	178 (5.41)	177 (5.61)	183 (11.2)	**0.90**
	10	178 (7.23)	172 (5.98)	180 (10.8)	**0.88**
	15	176 (4.31) ^{b}	179 (6.22) ^{ab}	185 (5.20) ^{a}	**0.04***
	p-values	**0.914**	**0.920**	**0.824**	
SCC ^{a} (x10^{3}/mL)	1	1640 (1523)	1977 (1554)	1678 (1534)	**0.62**
	10	2194 (1183)	1475 (1073)	902 (899)	**0.07**
	15	2480 (1420) ^{a}	1117 (949) ^{ab}	607 (593) ^{b}	**0.02***
	p-values	**0.42**	**0.24**	**0.15**	

*P ≤ 0.05 shows difference between groups (Note: means with the same lowercase letters do not differ).
+ P ≤ 0.05 shows the difference over time in each group (Note: averages with the same uppercase letters do not differ).
[a] Somatic cell count.

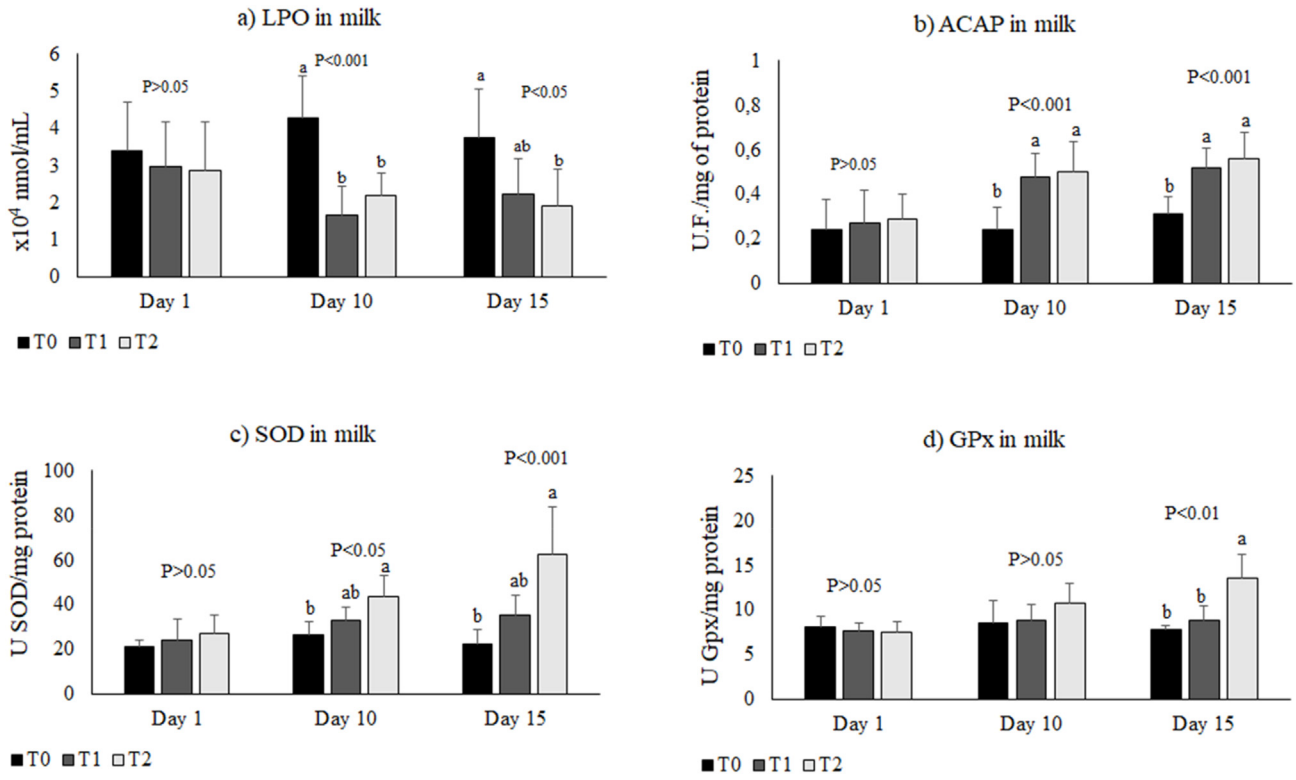

Fig. 1. Lipid peroxidation (LPO), total antioxidant capacity (ACAP), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in milk samples of dairy sheep. Treatments: no grape residue flour (GRF) supplementation (control group; CON; T0) or supply of 1% (1% GRF; T1) or 2% (2% GRF; T2) of GRF in the concentrate. P-values shows statistical differences between groups (same letters do not differ significantly).

Table 4
Hemogram of sheep not supplemented with grape residue flour (GRF), control group (CON) or supplemented with of 1% (1% GRF) or 2% (2% GRF) in the concentrate.

Variable	Day	CON	1% GRF	2% GRF	P-values
Erythrocytes (x10^6 μL)	1	5.14 (1.42)	5.15 (0.90)	5.05 (1.26)	**0.84**
	10	5.54 (1.64)	5.62 (1.41)	4.52 (0.65)	**0.08**
	15	5.80 (1.51)	5.26 (1.32)	4.61 (0.74)	**0.07**
	p-values	**0.35**	**0.41**	**0.74**	
Hematocrit (%)	1	28.0 (6.11)	29.3 (3.60)	30.1 (6.17)	**0.72**
	10	28.0 (5.84)	26.7 (3.41)	25.5 (5.32)	**0.60**
	15	29.1 (5.11)	27.9 (3.12)	29.0 (4.80)	**0.74**
	p-values	**0.84**	**0.78**	**0.65**	
Hemoglobin (mg/dL)	1	8.55 (1.82)	8.54 (1.41)	8.61 (1.60)	**0.90**
	10	8.81 (1.91)	8.22 (1.01)	7.43 (1.07)	**0.12**
	15	8.74 (1.73)	8.30 (0.96)	8.77 (1.02)	**0.41**
	p-values	**0.90**	**0.88**	**0.25**	
Leukocytes (x10^3 μL)	1	6.70 (3.67)	6.61 (1.81)	5.70^{A} (1.51)	**0.79**
	10	8.71 (3.94)^{a}	5.02 (1.40) ^{ab}	4.42^{AB} (0.76) ^{b}	**0.02**
	15	8.61 (1.71) ^{a}	4.91 (0.84) ^{b}	3.52^{B} (0.70) ^{c}	< **0.001**
	p-values	**0.40**	**0.29**	**0.03**$^{+}$	
Lymphocytes (x10^3 μL)	1	2.01 (1.21)	1.71^{A} (0.60)	1.83^{A} (0.74)	**0.75**
	10	2.42 (1.50)	0.96^{AB} (0.55)	1.04^{AB} (0.45)	**0.07**
	15	2.01 (0.94) ^{a}	0.92^{B} (0.17) ^{b}	0.70^{B} (0.36) ^{b}	< **0.001**
	p-values	**0.81**	< **0.001**	< **0.001**	
Neutrophils (x10^3 μL)	1	4.40 (2.33)	4.11 (1.32)	3.62 (0.91)	**0.55**
	10	5.92 (3.34)	3.90 (1.30)	3.21 (0.74)	**0.06**
	15	5.97 (1.20) ^{a}	3.63 (0.81) ^{b}	2.52 (0.61) ^{b}	< **0.001**
	p-values	**0.46**	**0.35**	**0.12**	
Monocytes (x10^3 μL)	1	0.19 (0.20)	0.07 (0.06)	0.09 (0.08)	**0.65**
	10	0.24 (0.18)	0.15 (0.19)	0.17 (0.11)	**0.70**
	15	0.51 (0.29)	0.28 (0.12)	0.23 (0.11)	**0.21**
	p-values	**0.56**	**0.60**	**0.65**	
Eosinophils (x10^3 μL)	1	0.07 (0.09)	0.12 (0.14)	0.09 (0.09)	**0.80**
	10	0.06 (0.08)	0.04 (0.05)	0.19 (0.36)	**0.52**
	15	0.17 (0.15)	0.03 (0.02)	0.02 (0.05)	**0.33**
	p-values	**0.50**	**0.74**	**0.40**	

*P ≤ 0.05 shows difference between groups (Note: means with the same lowercase letters do not differ).
+ P ≤ 0.05 shows the difference over time in each group (Note: averages with the same uppercase letters do not differ).

Table 5
Serum biochemistry of sheep not supplemented with grape residue flour (GRF; control group; CON) or supplemented with of 1% (1% GRF) or 2% (2% GRF) of GRF in the concentrate.

Variable	Day	CON	1% GRF	2% GRF	P-values
Glucose (mg/dL)	1	64.6^{AB} (11.6)	68.9^{AB} (7.50)	64.2^{B} (8.21)	**0.58**
	10	73.2^{A} (11.5)	80.8^{A} (11.7)	89.3^{A} (17.0)	**0.08**
	15	54.7^{B} (3.10)^{b}	57.0^{B} (7.21)^{ab}	66.6^{B} (7.3)^{a}	**0.03**
	p-values	< **0.001**	< **0.001**	< **0.001**	
Cholesterol (mg/dL)	1	91.8 (34.0)	95.3 (23.4)	127 (27.9)	**0.25**
	10	106 (34.1)	92.0 (20.2)	102 (16.1)	**0.43**
	15	69.9 (14.8)	62.0 (10.0)	78.5 (11.9)	**0.11**
	p-values	**0.20**	**0.12**	**0.22**	
Triglycerides (mg/dL)	1	23.3 (6.90)	27.1 (19.4)	22.1^{B} (5.60)	**0.40**
	10	17.2 (5.80)^{b}	18.0 (5.01)^{b}	31.2^{AB} (8.61)^{a}	< **0.001**
	15	20.1 (3.21)^{b}	26.9 (6.61)^{ab}	36.9^{A} (8.32)^{a}	< **0.001**
	p-values	**0.44**	**0.24**	< **0.001**	
Total protein (g/dL)	1	6.81 (0.95)	7.61 (1.01)	7.81^{AB} (0.61)	**0.15**
	10	8.74 (1.52)	8.62 (0.81)	9.30^{A} (0.82)	**0.69**
	15	7.64 (0.61)^{a}	7.32 (0.70)^{ab}	6.62^{B} (0.33)^{b}	**0.04**
	p-values	**0.26**	**0.25**	< **0.001**	
Albumin (g/dL)	1	2.50 (0.50)	2.71^{B} (0.32)	2.73^{B} (0.31)	**0.89**
	10	3.25 (0.42)	3.46^{A} (0.41)	3.62^{A} (0.62)	**0.62**
	15	2.91 (0.34)	2.82^{B} (0.21)	3.01^{AB} (0.32)	**0.80**
	p-values	**0.06**	**0.01**	< **0.001**	
Globulin (g/dL)	1	4.31 (0.41)	4.94 (0.91)	5.10^{A} (0.84)	**0.20**
	10	5.52 (1.20)	5.26 (0.81)	5.14^{AB} (1.90)	**0.76**
	15	4.76 (0.52)^{a}	4.59 (0.80)^{ab}	3.50^{B} (0.51)^{b}	**0.03**
	p-values	**0.339**	**0.552**	**0.010**	
Urea (mg/dL)	1	24.9^{B} (4.41)	26.7^{B} (8.11)	30.8 (6.60)	**0.18**
	10	42.2^{A} (5.60)	39.9^{AB} (12.1)	51.1 (18.3)	**0.36**
	15	38.0^{A} (3.91)^{a}	37.6^{A} (4.62)^{a}	27.6 (4.50)^{b}	< **0.001**
	p-values	< **0.001**	< **0.001**	**0.36**	

*P ≤ 0.05 shows difference between groups (Note: means with the same lowercase letters do not differ).
+ P ≤ 0.05 shows the difference over time in each group (Note: averages with the same uppercase letters do not differ).

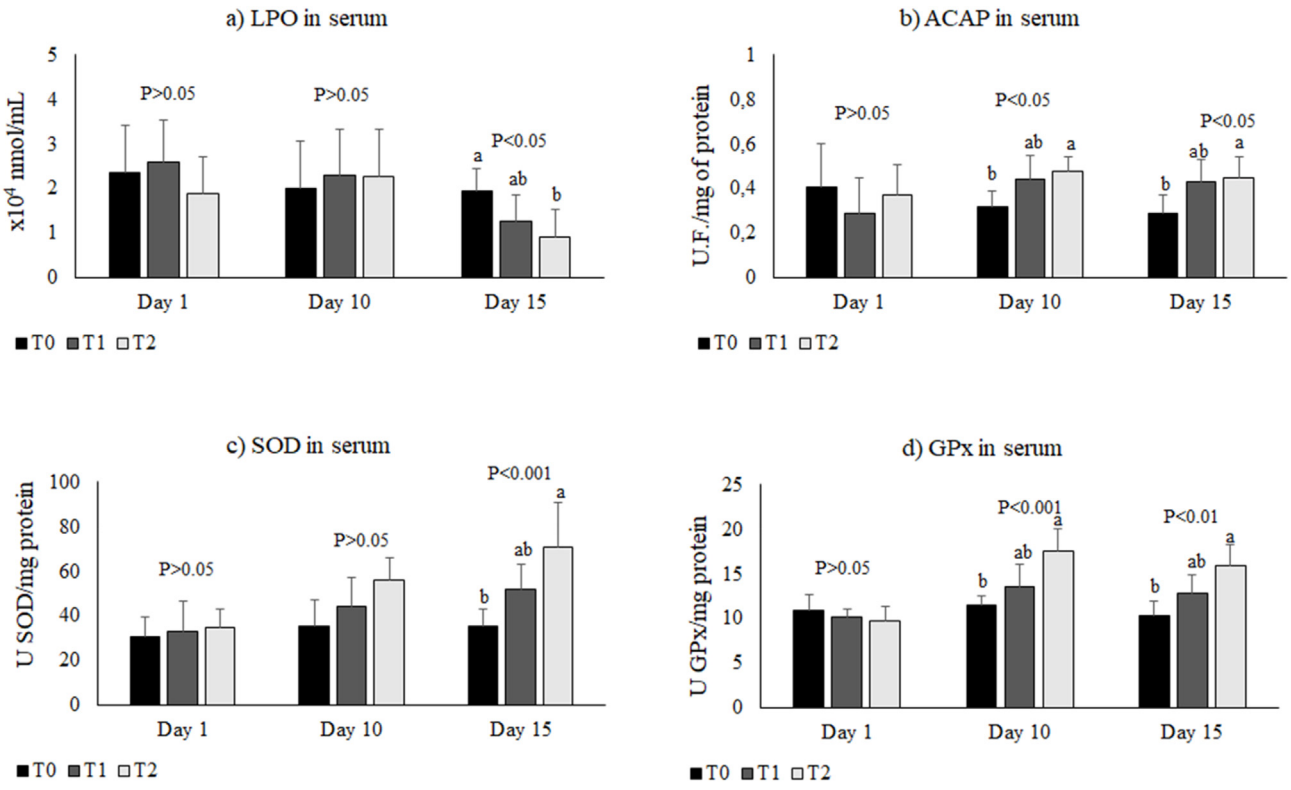

Fig. 2. Lipid peroxidation (LPO), total antioxidant capacity (ACAP), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in serum samples of dairy sheep. Treatments: no grape residue flour (GRF) supplementation (control group; CON; T0) or supply of 1% (1% GRF; T1) or 2% (2% GRF; T2) of GRF in the concentrate. P-values shows statistical differences between groups (same letters do not differ significantly).

15. Total protein and globulin concentrations were reduced ($P \leq 0.05$) in the 2% GRF group from d 1 to 15 (Table 5). Albumin concentrations did not differ ($P > 0.05$) between groups, but oscillated ($P \leq 0.05$) in the 1% GRF and the 2% GRF groups (Table 5). Urea concentrations were decreased ($P \leq 0.05$) in the 2% GRF group compared to 1% GRF and CON on d 15. Over time urea concentrations increased ($P \leq 0.05$) in CON and 1% GRF groups (Table 5).

3.7. Serum antioxidants and oxidants

Lipid peroxidation was reduced ($P \leq 0.05$) in the 2% GRF group compared to CON, and from d 10 to 15LPO was reduced ($P \leq 0.05$) in the 2% GRF group (Fig. 2A). Total antioxidant capacity was greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15, and from d 1 to 15 ACAP increased in the 1% GRF group (Fig. 2B). Superoxide dismutase activity was greater ($P \leq 0.05$) in the 2% GRF group compared to CON on d 15, and increased ($P \leq 0.05$) from d 1 to 10 and d 1 to 15 in the 1% GRF group (Fig. 2C). Activity of GPx was increased ($P \leq 0.05$) in the 2% GRF group compared to CON on d 10 and 15, and increased ($P \leq 0.05$) from d 1 to 10 and d 1 to 15 in the 1% GRF and 2% GRF groups (Fig. 2D).

3.8. Feed intake

Feed intake was not different ($P \leq 0.05$) between treatments. Sheep in all three groups consumed more than 95% of the diet provided daily and all concentrate was consumed.

4. Discussion

The increase in TPC and antioxidant activity in the diet with inclusion of GRF explains the elevated antioxidant response in the serum of sheep. Antioxidant activity (IC50) was not detected for the CON diet, suggesting that no chemical reactions were occurring that depend on antioxidants. Increased serum concentrations further support the increase in antioxidant capacity in the milk which has previously been observed in cows supplemented with grape residue silage (Santos et al., 2014). This effect is related to antioxidants, such as quercetin, resveratrol and phenols, that possess strong antioxidant capacity. According to Brower (1998), grape residues have nutraceutical actions when added to the diet because they possess medicinal compounds with beneficial health actions. In this study the inclusion of GRF in the diet minimized the oxidative stress in dairy sheep during heat stress.

Supplementation with GRF did not affect milk production (L), as observed by Santos et al. (2014) who provided grape residue silage for dairy cows. However, this study improved the productive efficiency of the sheep supplemented with GRF. As far as we know, this is the first study to show these effects of grape flour residue on efficiency and milk yield in dairy sheep. In the current study, the increase in productive efficiency with the inclusion of GRF in the diet may be a result of greater control of oxidative stress. Thus, improving the efficiency of nutrient utilization and increasing milk production numerically. Another hypothesis is that the increase in productive efficiency in sheep supplemented with GRF is due to the greater concentration of phenolic compounds and resveratrol which can positively influence immune function (Zunino and Storms, 2009; Cuevas et al., 2013), particularly in sheep with greater genetic potential for milk production.

In the current study, inclusion of GRF in diet (2% GRF) increased milk fat content. These findings disagree with previous results observed in dairy cows fed grape residue silage (Santos et al., 2014). This positive effect is believed to be related to an improvement in mammary gland function by reducing oxidative stress and decreasing amounts of free radicals (Celi, 2010). Total antioxidant capacity in milk was greater in sheep supplemented with GRF, which has been supported by previous results in dairy cows (Santos et al., 2014). Furthermore, Sánchez-alonso et al., 2007 reported that grape residues were able to reduce the

oxidation of stored fish meat and showed that oxidation was reduced in the first 90 days when compared to the control group. Therefore, improving the antioxidant activity in the milk of dairy sheep could lead to a reduction in lipid peroxidation. This reduction could potentially increase the shelf life of products such as milk, cheese, and yogurt when animals consume grape residues in the diet. However, shelf life was not evaluated in this study but will be an important variable in future studies performed in this laboratory.

It is important to point out the reduction on SCC in milk samples from 2% GRF sheep. It is well understood that SCC includes cells of sanguine origin (leukocytes) and desquamation of the secretory glandular epithelium (Jorge et al., 2005) A reduction in SCC is linked to the systemic anti-inflammatory capacity caused by the components present in the GRF. This anti-inflammatory effect was confirmed by a reduction in the number of lymphocyte and globulin concentrations. These results are similar to those reported in dairy sheep fed a diet supplemented with curcumin (Jaguezeski et al., 2018). While there was a reduction in the number of leukocytes, values remained within the normal range for adult sheep throughout the 15-day study. However, it important to emphasize the need for future studies in order to evaluate the long-term impacts on the immune system, especially in animals suffering from heat stress.

Lactation is a critical period in sheep and cows because they suffer from greater oxidative stress. According to Barbosa et al. (2008), some nutrients (vitamins A, B, C, as well as resveratrol, quercetin, etc.) are beneficial to an animal's health by reducing the degree of oxidative stress. In this regard, addition of GRF for lactating sheep resulted in an increase in total antioxidant capacity which is linked to an upregulation in the enzymes involved in the antioxidant system, including, SOD and GPx. Similarly, addition of grape residues in broiler chicken feed increased the antioxidant levels in the muscle at the same proportion as animals supplemented with vitamin E (Brenes et al., 2008). Suggesting that grape residues can be considered a probable substitute for vitamins currently supplied in feed. The decrease in LPO and increase in SOD enzyme activity in sheep receiving 2% GRF are similar to the results found by Megahed et al. (2008), where the application of vitamin E and selenium (substances with antioxidant action) resulted in a decrease in LPO and nitric oxide concentration and an increase in SOD enzyme activity, which resulted in a reduction of oxidative stress in buffaloes under heat stress.

According to Kang et al. (2005), the presence of oxidative stress signals stimulates Nrf-2 activity, which then stimulates the expression of genes that results in the production of antioxidant enzymes, such as GPx. The increase in GPx activity was more pronounced in animals supplemented with 2% GRF. Similarly, Janiques et al. (2014) observed an increase on GPx activity in humans supplemented with grape residues. These findings could be explained by the presence of polyphenols in grape residues, Polyphenolic compounds stimulate the activity of Nrf-2 and upregulate the expression of antioxidant genes (Ghanim et al., 2011). Thus, the increase in SOD and GPx activity can be related to the stimulation on Nrf-2.

Another factor that may influence the imbalance between antioxidants and oxidants is heat. Sheep undergoing heat stress have an increased respiration rate (Filho et al., 2011), which was also observed in the current study. Increased respiration rates were observed in the afternoon, when sheep also exhibited decreased physical and alimentary activity, which according to McDowell (1989), can impair food intake and the rumination process, reducing performance and animal production. Variation in seasonal temperatures alters the concentrations of hormones and oxidative markers in buffaloes (Megahed et al., 2008). Authors found that the concentration of estradiol and the enzyme superoxide dismutase (SOD) was decreased in the summer compared to winter while LPO and nitric oxide (ON) were significantly higher in the summer compared to the winter Filho et al. (2011), observed that Santa Inês lambs were in greater thermal comfort at temperatures between 10 °C and 25 °C, reinforcing that sheep were under heat stress during the current experiment, where the maximum temperatures recorded were 27 °C to 35 °C, respectively. Even with the effect of temperature, the 2% GRF group had an increase in total antioxidant capacity in both serum and milk, which is beneficial for the animals.

In this study, serum levels of urea and glucose were reduced while serum triglyceride concentrations were greater in animals supplemented with 2% GRF. However, when dairy cows were supplemented with grape residue silage, these variables were not altered (Santos et al., 2014). In the current study, digestibility was not evaluated but the study conducted by Santos et al. (2014) revealed that grape residue provided in the diet of dairy cows led to a decrease in the content of nutrients, dry matter, crude protein, ethereal extract and fibers. Similar results were observed in sheep fed a dehydrated wine residue which consisted of different energy sources (Barroso et al., 2006). It is believed that a reduction in digestibility can be related to lower serum levels of glucose and urea and potentially reducing protein and carbohydrate metabolism. The increase in triglycerides could be a consequence of increased amounts of ethereal extract in the 2% GRF diet.

5. Conclusion

Addition of grape residue flour in the diet of dairy sheep increased the levels of total phenolic compounds and antioxidant activity in the concentrate. Increased antioxidants in the diet stimulated the antioxidant response in the sheep in heat stress and reduced oxidative stress. Furthermore, there was an increase in antioxidant capacity in milk which was associated with lower lipid peroxidation. Also, supplementation with 2% grape residue flour improved sheep health and productive efficiency. We concluded that grape residue flour supplementation generates an anti-inflammatory response which has a positive effect on milk quality because it reduces somatic cell count in milk samples. Thus, grape residue supplementation improves health, milk performance and milk quality.

Ethical note

The study was approved by the Committee of Ethics in the Use of Animals of the State University of Santa Catarina (CEUA/UDESC) under number 5184250218.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.04.007.

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