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ANIMAL SCIENCE

Microencapsulated herbal components in the diet of Lacaune ewes: impacts on physiology and milk production and quality

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Abstract: This study aimed to determine whether the addition of a microencapsulated herbal blend (MHB) based on thymol, carvacrol, and cinnamaldehyde in dairy sheep feed would improve production efficiency, milk quality, and animal health. Thirty lactating Lacaune ewes were divided into three groups: Control (T0), 150 mg blend/kg of feed (T150), and 250 mg blend/kg of feed (T250). Milk was measured before the beginning of the experiment (d 0), at the end of the adaptation period (d 15), and during the experiment (d 20). In milk samples, was measured the composition, somatic cell count (SCC), reactive oxygen species (ROS), lipoperoxidation (LPO), and total antioxidant capacity. The MHB improved the milk production (only T150 vs. T0 sheep on d 20), productive efficiency and feed efficiency, and reduced the milk SCC (only T250 vs. T0 sheep, on d 20), ROS and tended to reduce the milk levels of LPO (only T250 vs. T0 sheep on d 20). Also, MHB reduced the blood levels of neutrophils and ROS (only T250 vs. T0 sheep on d 20) and increased total protein and globulin levels. Thus, a microencapsulated blend of thymol, carvacrol, and cinnamaldehyde improved the productive performance and milk quality of sheep.

Key words: feed efficiency, antioxidants, animal health, productivity, nutrition.

INTRODUCTION

Sheep's milk is consumed in various parts of the world; however, the world production of sheep's milk is small compared to that of other ruminants: 11,811,328 tons in 2018 (FAOSTAT 2018). According to FAO, this production is led by Turkey (1,446,271 tons), followed by China (1,180,276 tons), and Greece (753,819 tons) (FAO STAT 2018). A sheep's lactation period lasts an average of 150 days (Brito et al., 2006), a relatively short cycle compared to that of cows that produce for 305 days (Jonas et al. 2011). The sheep's lactation peak is close to 30 days postpartum. It can produce about 4.5 L of milk daily during this period; however, after the production peak, the average daily production drops to 1.3 L (Brito et al. 2006). Lacaune ewes have production 30% greater than of the East Friesian breed at the beginning of the lactation period; however, the decline in production is faster over time, reaching a decrease of about 8 g in daily production, while the East Frisian loses 2 g (Ticiani et al. 2013).

Essential oils have been studied as modifiers of ruminal fermentation through the modulation of their microbiota to reduce energy losses during digestion and convert energy to milk or meat production (Calsamiglia et al. 2007, Soltan et al. 2018). The favoring of gram-negative bacteria results in the production of succinate

and degradation of lactate in the rumen, which modulates rumen pH. These additives stimulate the formation of propionic acid and reduce methanogenesis and proteolysis, and deamination of dietary protein in the rumen (Nicodemo 2001, Soltan 2018). According to the literature, essential oils can be extracted from aromatic plants using various methodologies, including steam distillers, hydrodistillation, liquid carbon dioxide, or microwaves (Bakkali et al. 2008). The active components present in oils can be found in buds, stems, flowers, fruits, seeds, fruit branches, roots, or even bark (Bakkali et al. 2008).

Most research on this subject has focused on thymol and carvacrol, compounds extracted from thyme, oregano, and cinnamon, with varying chemical structures: 5-methyl-2-(1-methylethyl)-phenol, 2-methyl-5-(1-methylethyl)-phenol, and 3-phenyl-2-propenal phenol, respectively (Calsamiglia et al. 2007, Nostro & Papalia 2012). This interest is related to the mechanism of action thymol and carvacrol that act on the lipid bilayer of bacteria, altering their conformation and causing destabilization and extravasation of cellular content (Calsamiglia et al. 2007). In an attempt to compensate using electric pumps, bacteria expend large amounts of energy and reduce their growth capacity (Calsamiglia et al. 2007, Bakkali et al. 2008, Silva et al. 2012). This property allows stabilization of the rumen environment and improves the use of nutrients by ruminants, and protects against the invasion of pathogenic microorganisms, positively impacting animal health and production (Nicodemo 2001). Thymol and carvacrol modulated the immune system, in addition to having anti-inflammatory, antioxidant, analgesic, and spasmolytic effects (Bakkali et al. 2008, Lima et al. 2013). In ruminants, there was a stimulation of antioxidant activity and decreased lipid oxidation in the meat of steers that consumed essential oils containing thymol (Monteschio et al. 2007). Cinnamaldehyde can modify nitrogen metabolism, decreasing the concentrations of ammonia and volatile fatty acids (Cardozo et al. 2004, Busquet et al. 2006), and when cinnamaldehyde associated with thymol and carvacrol, it increased milk production in multiparous cows (Wall et al. 2014).

Based on herbal compounds, commercial products have been called phytogenics or feed additives (Windisch et al. 2008). In ruminant feed, the use of phytogenics has encountered limitations, with emphasis on losses during the passage through their multicavity stomach, as well as degradation by ruminal fermentation (Oliveira et al. 2013); just as we cannot rule out adverse effects of fermentative bacteria. Knowing these problems, researchers developed the microencapsulation process of herbal components, which promotes stability to the phytogenic and releases slowly and under specific conditions (Pereira et al. 2018). According to the literature, the use of a microencapsulated phytogenic also allows the herbal components to be protected from the action of ruminal bacteria, reducing their loss and allowing greater absorption in the intestine (Shen et al. 2017), as the raw material used for microencapsulation it is regulated by pH, that is, the microcapsule opens only at intestinal pH (Laurenti & Garcia 2013). Microencapsulated herbal components are a new technology used in the feeding of ruminants. Therefore, the objective of this study was to evaluate whether the addition of a blend of microencapsulated herbal components in the diet of dairy sheep has positive effects on production efficiency, milk quality, and animal health.

MATERIALS AND METHODS

This experiment was carried out following animal welfare practices and approved by the Ethics Committee for the Use of Animals in Research (CEUA / UDESC), protocol number 7308030419.

Phytogenic

We used a microencapsulated herbal blend - MHB (Enterosan®, Konkreta, Brazil) in our experiment. The commercial product was analyzed to evaluate the guaranteed levels. Our colleagues, Galli et al. (2020) previously published these data: 21.55 mg of carvacrol, 18.76 mg of thymol, and 27.62 mg of cinnamaldehyde per gram of phytogenic agent.

Animals and experimental design

The experiment was carried out on a commercial sheep farm located in Chapecó, Santa Catarina, Brazil. The experiment lasted 20 d, with the first 15 d involving diet adaptation. Thirty multiparous Lacaune ewes (3rd delivery) were selected, with an average body weight of 68 ± 3.8 kg and an average of 50 ± 3.0 d of lactation. The animals were stratified by milk production and then randomly assigned to one of three pens. The stalls (24 m^2) were in a covered shed, with no walls and a hard floor with a wood bed. The feeders were divided, making it possible to match the feed supply to the animals individually. That is, the animals were confined during feeding (feed consumption was measured individually).

The groups formed were as follows: without the addition of phytogenic, used as a control (T0); 150 mg MHB/kg of feed (T150); and 250 mg MHB/kg of feed (T250).

In the collective stalls, the sheep were trapped in a bowl in their feeders immediately after milking. Each animal received 1.2 kg/d of concentrate, divided into two daily feedings (0700h and 1700h). The concentrate was offered first and approximately 15 min. Later, every day of the experiment, 100% of the concentrate supplied to the sheep was consumed.

Then, approximately 4.0 kg/d of corn silage (green matter) was divided into three daily feedings (0700h; 1100h, and 1700h) (Table I). The sheep were contained using a headlock to their feeder for 1 h, thereby guaranteeing individual consumption of silage. On days 16, 17, 18, 19, and 20, silage intake also was measured by subtracted orts weight from the amount of feed offered daily. After feed consumption, the animals remain at rest, free in the collective stall, with free access to water until the next feed or milking.

Analysis of the chemical composition of the diet

Samples of silage and concentrate were collected (days 1, 12, and 20 of the experiment) for analysis of the chemical composition, stored freezing (–20 ºC) until analysis (Silva & Queiroz 2002). Before the analysis, a pool of these three samples was performed, processed, and analyzed in triplicate. The concentrate was ground in a hammer-type mill with a grain size of 1 mm; forage was ground using a knife mill.

The feed samples were 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 non-fibrous carbohydrates (NFC) were calculated according to NRC (2001): NFC (%) = 100 – (% NDF + % CP + % EE + % ash). The concentration of neutral detergent fiber (NDF) and acid (ADF) were performed according to the methodology of Van Soest et al. (1991; without the addition of sodium sulfite and alpha-amylase). Results are presented in Table I.

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

Ingredients	As fed (kg/day)	Dry matter (DM; kg/day)		
Corn silage (kg)	4.0	1.32		
Concentrate (kg)	1.2	1.05		
Chemical composition	Corn silage	Concentrate (T0)	Concentrate (T150)	Concentrate (T250)
DM, g/kg	329.2	879	881.6	882.4
Ash, g/kg DM	42.1	60.2	64.0	62.0
CP, g/kg DM	86.0	170.6	175.2	174.8
NDF, g/kg DM	333.0	95.6	82.0	84.0
ADF, g/kg DM	178.6	44.0	29.5	34.8
EE, g/kg DM	46.6	44.4	37.1	38.7
NFC, g/kg DM	492.3	629.2	641.7	640.5
TPC (mg GAE/100 g DM)	-	0.006	0.003	0.017
IC_{50} (mg/mL)	-	3.93	2.83	2.58

Ingredients present in 100 kg of concentrate: corn (70%), soybean meal (25%) and buffering lactation nucleus (5%), i.e., 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 min. 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 IU; monensin sodium 1200 mg; *Saccharomyces cerevisiae* 2.1 x 10^{10} CFU).

[2] Note: DM (Dry matter), CP (Crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), EE (ethereal extract) and NFC (non-fibrous carbohydrates).

[3] Note: Total phenolic compounds (TPC: mg GAE/100 g DM); Antioxidant activity against DPPH - IC_{50} radical (mg/mL)

Milk measurement

The measurement of milk production of the animals was made on d 0, 15 to 20, using a "Milk Meter" (True Test® meter, Auckland, New Zealand). The average milk production between days 16 to 20 was calculated, and the results were presented as being at d 20 of this study. The total production value was the result of the sum of the two daily milkings.

The productive efficiency (%) was calculated according to a methodology described by Alba et al. (2019), based on the difference between milk production on d 15 and 20 of the experiment with that of d 0. Feed conversion was calculated based on the formula: daily feed consumption/daily milk production. The feed efficiency index (IEA) was calculated as the average production of animals in each group divided by the average consumption of feed per animal (Souza 2003).

Sample collection

Blood and milk collections were performed on d 0, 15, and 20 of the experiment, at 0700h, with the animals fasting. Restraint was performed manually, using vacuum tubes (4 mL per animal). Blood was collected through puncture of the jugular vein. One of the tubes contained ethylenediamine tetra-acetic acid and was used to collect blood for complete blood counts. Blood smears; the other contained clot activator (silica) and was used to obtain the serum for serum biochemical and oxidant/antioxidant analyses. Blood samples with clot activator were centrifuged at 3,800 *g* for 10 min, and after separation, the serum was pipetted and placed in microtubes and subsequently frozen (–20 ºC) until analysis.

The milk samples were collected during the first milking of the day, using a "Milk Meter" type meter (Tru Test®) for the homogeneous sampling

of milk production. The samples were stored in isothermal boxes with ice in a reusable gel (4 ºC) and transported to the laboratory, where they were processed.

Hemogram

Red blood cell counts, total leukocyte counts, and hemoglobin concentrations were measured using the semi-automatic analyzer (CC-530 CELM). The leukocyte differential was performed using blood smears stained with a commercial kit (Panotic Rapid, Laborclin). Cell identification was performed using an optical microscope (100x). The hematocrit was obtained after capillary centrifugation at 10,000 rpm for 5 min (Feldman et al. 2000).

Serum biochemistries

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) activity were evaluated, as well as the levels of total proteins (PT), albumin, urea, triglycerides, cholesterol, and glucose. Measurements were performed using a semi-automatic analyzer (BioPlus 2000®), using specific commercial kits (Analisa®, Gold Analisa Diagnóstica, Belo Horizonte, Brazil). Globulin levels were calculated as the difference between albumin and total protein levels.

Oxidants and antioxidants in serum and milk

Superoxide dismutase (SOD) activity was measured using the auto-oxidation principle of pyrogallol (inhibition in the presence of SOD) with a kinetic evaluation of the optical density at 420 nm for two minutes at ten-second intervals and was expressed as U SOD/mg protein (Beutler 1984). Non-protein thiol levels (NPSH) were measured according to Sedlak & Lindsay (1968). Levels of ROS were obtained after incubation of 10 µL of serum, added in 12 µL dichlorofluorescein in 1 mm at 37 °C for 1 h in the dark (Ali et al. 1992); 488 nm was used for excitation and 520 nm for emission to determine fluorescence and the results were expressed as U DCF/mL. Levels of lipid peroxidation (LPO) were determined according to Monserrat et al. (2003), and the results were expressed as µmol CHP/mL. The analysis of antioxidant capacity against peroxyl radicals (ACAP) was carried out according to Amado et al. (2009), and the results were expressed as U fluorescence/mg protein.

Proximate composition and milk quality

An automatic infrared analyzer (LactoStar Funke Gerber®) was used to determine the concentrations of proteins, lactose, fat, and total solids. Somatic cell counts (SCC) were measured using semi-automatic equipment (Ekomilk Scan Somatic Cells Analyzer®).

Statistical analysis

Each animal was considered the experimental unit for all analyses. All dependent variables were tested for normality using the Univariate procedure of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4), and all were normally distributed. Then, all data were analyzed using the MIXED procedure of SAS, with the Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. Productive efficiency, feed intake, feed conversion, and feed efficiency were tested for fixed effect of treatment using animal (treatment) as random effects. All other study variables were analyzed as repeated measures and tested for a fixed effect of treatment × day. The subject used was animal(treatment). The compound symmetric covariance structure was selected for milk production; the Toeplitz covariance structure was selected for hematocrit, neutrophils, and eosinophils; and the first-order autoregressive covariance structure was selected for all other variables. The covariance structures were selected

according to the lowest Akaike information criterion. Means were separated using PDIFF, and all results were reported as LSMEANS followed by SEM. Significance was defined when $P \leq 0.05$.

RESULTS

Milk performance

The results of milk performance are presented in Table II. Effect of treatment × day ($P = 0.01$) was detected for milk production. T150 ewes had significantly greater milk production only on d 20, compared to T0 ewes. T150 and T250 ewes had significantly greater ($P \leq 0.04$) productive efficiency and feed efficiency than T0 ewes. Although feed intake was not affected by treatments ($P = 0.21$), T250 ewes had significantly greater ($P = 0.05$) feed conversion when compared to T0 ewes.

Milk composition and quality

The results of milk composition and quality are presented in Table III. Effects of treatment × day were not detected for milk concentration of protein, fat, lactose, total solids, and ACAP. Effects of treatment × day were detected ($P = 0.05$) for SCC in milk. T250 ewes have significantly less SCC on d 20 compared to T0. Effects of treatment × day and treatment were detected for milk concentration of ROS ($P = 0.01$), i.e., T150 and T250 ewes had significantly lower concentrations on d 15 and 20 when compared to T0 ewes; as well as a tendency to reduce LPO ($P = 0.10$).

Hemogram

The results of the hemogram are presented in Table IV. Effects of treatment × day were not detected ($P \geq 0.11$) for hematocrit, erythrocytes, hemoglobin, lymphocytes, monocytes, or eosinophils. T250 vs. T0 ewes had significantly fewer leukocytes and neutrophils on d 15, and T150 and T250 vs. T0 ewes had significantly fewer of these cell types on d 20.

Table II. Milk performance of Lacaune ewes supplemented with dietary phytogenic.

Variables	Treatments[1]			SEM	*P*-value	
	T0	T150	T250		Treatment	Treatment × day
Production (L)					-	0.01
d 0	2.22	2.21	2.21	0.10		
d 15	2.18	2.14	2.26	0.11		
d 20[2]	2.25[b]	2.49[a]	2.35[ab]	0.09		
Productive efficiency (%)						
d 0 to 15	0.93[c]	4.56[b]	8.41[a]	1.81	0.01	
d 0 to 20	4.18[b]	13.6[a]	10.3[a]	2.11	0.01	
Feed intake/animal (%)						
d 15 to 20	84.1	90.0	85.2	7.74	0.21	
Feed conversion						
d 15 to 20	0.85[a]	0.82[ab]	0.81[b]	0.01	0.05	
Feed efficiency						
d 15 to 20	117.1[b]	122.8[a]	122.6[a]	2.14	0.04	

[a-c]**Differs ($P \leq 0.05$) between treatments each respective day.**
[1]**T0, T150, and T250 represent 0, 150, and 250 g of phytogenic/kg of concentrate, respectively.**
[2] **Average milk production between days 16 to 20 was calculated, and the results were presented as being at d 20 of this study.**

Table III. Milk composition and quality of Lacaune ewes supplemented with dietary phytogenic.

Variables[1]	Treatments[2]			SEM	*P*-value
	T0	T150	T250		Treatment × day
Milk composition					
Protein (g/kg)					0.87
d 0	5.92	5.85	5.88	0.10	
d 15	5.87	5.81	5.79	0.12	
d 20	5.91	5.86	5.96	0.11	
Fat (g/kg)					0.88
d 0	5.97	5.62	5.50	0.25	
d 15	6.01	5.96	5.74	0.24	
d 20	5.86	6.02	5.85	0.23	
Lactose (g/kg)					0.87
d 0	5.56	5.59	5.62	0.13	
d 15	5.55	5.53	5.60	0.14	
d 20	5.65	5.47	5.57	0.15	
Total solids (g/kg)					0.59
d 0	17.45	17.06	17.00	0.42	
d 15	17.43	17.30	17.13	0.39	
d 20	17.42	17.35	17.38	0.40	
Milk quality					
SCC[1] (x10^3 /mL)					0.05
d 0	151.30	143.80	156.10	29.25	
d 15	151.00	131.00	154.40	29.22	
d 20	216.30[a]	175.90[ab]	150.20[b]	29.16	
ROS[1] (U DCF/mg protein)					0.01
d 0	0.66	0.64	0.65	0.55	
d 15	8.37[a]	4.53[b]	4.44[b]	0.59	
d 20	9.15[a]	4.54[b]	4.72[b]	0.49	
LPO[1] (µmol CHP/ mL)					0.10
d 0	395.3	635.7	588.4	144.4	
d 15	412.4	452.7	398.7	106.7	
d 20	432.9[a]	387.7[ab]	354.8[b]	98.71	
ACAP[1] (UF/ mg protein)					0.15
d 0	0.71	0.65	0.67	0.03	
d 15	0.66	0.74	0.71	0.05	
d 20	0.64	0.79	0.82	0.03	

[1] Somatic cell count (SCC), reactive oxygen species (ROS), lipid peroxidation (LPO), and antioxidant capacity against peroxyl radicals (ACAP).
[2] T0, T150, and T250 represent 0, 150, and 250 g of phytogenic/kg of concentrate, respectively.
[a-b] Differs ($P \leq 0.05$) between treatments each respective day.

Serum biochemistry

The results of serum biochemistry are presented in Table V. There were effects of treatment × day for serum concentration of glucose, albumin, triglycerides, urea, AST, and ALT. However, effects of treatment × day ($P = 0.05$) were detected for serum concentrations of total protein, and T150 and T250 ewes had significantly greater concentrations of total protein on d 15 and 20, compared to T0 ewes. Effects of treatment × day were detected ($P \leq 0.03$) for serum concentration of globulin; T150 ewes had significantly greater globulin concentrations on d 15; T150 and T120 ewes had significantly greater concentrations on d 20 when compared to T0 ewes. Effects of treatment x day ($P = 0.05$) were detected for serum

Table IV. Hemogram of Lacaune ewes supplemented with dietary phytogenic.

Variables	Treatments[1]			SEM	*P*-value
	T0	T150	T250		Treat × day
Hematocrit (%)					0.33
d 0	31.1	30.9	32.3	0.99	
d 15	31.8	32.7	33.7	0.87	
d 20	31.5	32.8	33.9	0.96	
Erythrocytes (x10^6µL)					0.42
d 0	7.99	8.00	8.15	0.29	
d 15	8.14	8.24	8.16	0.27	
d 20	8.05	8.19	8.68	0.21	
Hemoglobin (g/dL)					0.21
d 0	9.81	9.76	9.87	0.27	
d 15	9.96	9.84	9.65	0.27	
d 20	9.67	9.74	10.2	0.24	
Leukocytes (x10^3 µL)					0.04
d 0	21.2	21.0	20.9	3.13	
d 15	22.4[a]	18.6[ab]	15.0[b]	3.01	
d 20	22.6[a]	16.1[b]	16.0[b]	2.97	
Neutrophils (x10^3/µL)					0.02
d 0	10.6	9.78	10.6	1.52	
d 15	14.1[a]	10.6[ab]	7.41[b]	1.62	
d 20	13.9[a]	8.24[b]	8.90[b]	1.73	
Lymphocytes (x10^3/µL)					0.31
d 0	8.97	9.73	8.92	0.80	
d 15	7.45	7.06	6.74	0.74	
d 20	7.96	7.01	6.82	0.85	
Monocytes (x10^3/µL)					0.20
d 0	0.15	0.15	0.12	0.03	
d 15	0.15	0.10	0.17	0.07	
d 20	0.10	0.12	0.15	0.05	
Eosinophils (x10^3/µL)					0.11
d 0	1.54	1.40	1.34	0.22	
d 15	0.74	0.84	0.76	0.17	
d 20	0.69	0.74	0.67	0.19	

[1]T0, T150, and T250 represent 0, 150, and 250 g of phytogenic/kg of concentrate, respectively.
[a-b]Differs ($P \leq 0.05$) between treatments each respective day.

concentration of cholesterol, i.e., T150 ewes had significantly greater concentrations than T0 ewes. Effects of treatment × day and treatment were detected ($P = 0.02$) for serum activity of GGT; because T150 ewes had significantly greater concentrations only on d 15, compared to T0 and T250 ewes.

Table V. Serum biochemistry variables of Lacaune ewes supplemented with dietary phytogenic.

Variables[1]	Treatments[2]			SEM	*P*-value
	T0	T150	T250		Treat × day
Glucose (mg/dL)					0.17
d 0	55.2	53.8	53.3	2.20	
d 15	62.4	66.7	69.4	2.21	
d 20	60.4	67.4	66.7	2.34	
Total Protein (g/dL)					0.05
d 0	6.67	6.51	6.65	0.46	
d 15	7.97[b]	9.84[a]	9.02[a]	0.43	
d 20	7.61[b]	9.63[a]	9.23[a]	0.43	
Albumin (g/dL)					0.40
d 0	3.14	3.25	3.08	0.12	
d 15	3.37	3.96	3.73	0.14	
d 20	3.50	3.61	3.14	0.15	
Globulin (g/dL)					0.03
d 0	3.53	3.26	3.57	0.39	
d 15	4.60[b]	5.88[a]	5.29[ab]	0.37	
d 20	4.11[b]	6.02[a]	6.09[a]	0.37	
Cholesterol (mg/dL)					0.05
d 0	85.6	82.7	84.5	5.01	
d 15	87.9	98.4	99.1	4.25	
d 20	86.0[b]	101.7[a]	95.1[ab]	4.24	
Triglycerides (mg/dL)					0.39
d 0	23.0	22.2	20.83	0.96	
d 15	22.4	22.9	21.7	0.87	
d 20	22.7	23.4	24.3	0.64	
Urea (mg/dL)					0.30
d 0	58.6	62.4	56.1	2.42	
d 15	54.3	56.8	57.4	2.43	
d 20	55.1	53.8	56.7	2.08	
AST (U/L)					0.64
d 0	104.8	110.7	114.3	7.96	
d 15	99.7	106.0	101.3	8.97	
d 20	109.9	106.7	93.0	8.53	
ALT (U/L)					0.93
d 0	13.8	14.9	13.3	0.86	
d 15	14.7	11.9	12.7	0.95	
d 20	13.0	14.1	12.3	0.75	
GGT (U/L)					0.02
d 0	56.9	63.0	59.5	7.68	
d 15	106.3[b]	147.7[a]	105.0[b]	7.32	
d 20	105.5	113.8	100.4	7.32	

[1]**Aspartate aminotransferase (AST), Alanine transaminase (ALT), and gamma-glutamyl transferase (GGT).**
[2]**T0, T150, and T250 represent 0, 150, and 250 g of phytogenic/kg of concentrate, respectively.**
[a-b]**Differs (*P* ≤ 0.05) between treatments each respective day.**

Oxidant/antioxidant status

The results of serum oxidants/antioxidants variables are presented in Table VI. Effects of treatment × day were detected (P = 0.01) for serum concentration of ROS, and T250 ewes had significantly lower concentrations only on d 20, compared to T0 ewes. Effects of treatment × day were not detected (P ≥0.18) for serum levels of LPO, and NPSH, as well as for SOD activity.

Table VI. Serum oxidants/antioxidants variables of Lacaune ewes supplemented with dietary phytogenic.

Variables[1]	Treatments[2]			SEM	*P*-value
	T0	T150	T250		Treat × day
ROS[1] (U DCF/mg protein)					0.01
d 0	0.40	0.52	0.56	0.09	
d 15	0.48	0.42	0.45	0.09	
d 20	0.64[a]	0.51[ab]	0.33[b]	0.09	
LPO[1] (µmol CHP/mL)					0.13
d 0	72.9	80.2	92.0	6.90	
d 15	70.6	74.3	79.1	5.41	
d 20	71.8	67.9	60.7	6.85	
SOD[1] (U SOD/mg protein)					0.36
d 0	5.72	5.51	5.61	0.11	
d 15	5.03	5.17	5.21	0.09	
d 20	5.41	5.32	5.68	0.13	
NPSH[1] (µmol/mL)					0.36
d 0	1.89	1.82	1.86	0.04	
d 15	1.74	1.69	1.81	0.09	
d 20	1.91	1.84	1.90	0.10	

[1]**Reactive oxygen species (ROS), lipid peroxidation (LPO), superoxide enzyme dismutase (SOD), and non-protein thiols (NPSH).**
[2]**T0, T150, and T250 represent 0, 150, and 250 g of phytogenic/kg of concentrate, respectively.**
[a-b]**Differs (*P* ≤ 0.05) between treatments each respective day.**

DISCUSSION

The animals that received 150 g of phytogenic/kg of concentrate had a greater volume of milk produced than the no inclusion group. This suggests that the use of a phytogenic based on thymol, carvacrol, and cinnamaldehyde improved production. Such results can be explained by the capacity of these compounds to modify rumen fermentation and cause increases in productive performance, owing to their bactericidal, antiparasitic, and antioxidant activities, in order to modify the microorganisms that are part of digestion, in addition to preventing the action of free radicals on the DNA of cells involved in food absorption (Alagawany et al. 2015). Maenner et al. (2011) showed that the addition of essential oils to piglet feed improved performance, with improvement in the feeding efficiency, mainly associated with the ileal digestibility of the amino acids (6.5% improvement) and CP (6% to 12% improvement). It is possible to modulate ruminal fermentation through the control of proliferation and inhibition of certain ruminal bacteria. Phenolic substances such as thymol and carvacrol interact with bacterial membranes, killing them through the overflow of ions with a slower replacement and causing energy expenditure that overwhelms the amount of energy required for bacterial growth. Microencapsulated essential oils composed of carvacrol, eugenol, and cinnamaldehyde were supplied to sheep, increasing propionate production; the investigators found a decrease in protozoa, suggesting that the growth of propionate-producing microorganisms may be favored by these compounds (Soltan et al. 2018). Propionate is used for the synthesis of glucose and galactose that results in lactose production. Lactose from propionate acts on the mammary gland, increasing milk production (Alves Filho

2005). Recently, Benchaar (2020) found that the inclusion of 50 mg of carvacrol/kg feed for 30 d did not have significant effects on the performance of milk production in dairy cows, nor did it alter the ruminal fermentation or improve the utilization of nutrients; the authors concluded that this dose was ineffective as a supplement to increase milk production. In the present study, 150 mg carvacrol/kg feed with the other herbal components increased milk production. In particular, there was a possible dose-dependent effect of carvacrol on milk production, a synergistic effect with the other herbal components, or an effect of the microencapsulated form of this carvacrol.

Lower ROS were found simultaneously with the decrease in the number of neutrophils. Neutrophils form an essential line of defense, with the ability to engulf pathogens and foreign particles and eliminate or inactivate them. During the elimination of these pathogens, the production of ROS occurs through cytoplasmic organelles of neutrophils. ROS play important bactericidal and bacteriostatic roles; however, their great oxidative potential damages tissues when produced in excess and over long periods, evolving into degenerative diseases (Kielland et al. 2009, Silva 2015). This leads us to suggest that the significant decrease in LPO and ROS levels in milk and ROS in the blood may result from the drop in the number of neutrophils that occurred on the same day in animals that received the phytogenic (Table IV). Aristatile et al. (2015) found that carvacrol inhibited the formation of free radicals, decreased the concentration of TBARS and LHP, and maintained high levels of vitamin C and vitamin E in human neutrophils exposed to UVB radiation, in addition to significantly reducing DNA damage, promoting protection against oxidative stress. Cabello et al. (2015) demonstrated that carvacrol had dose-dependent antioxidant activity, i.e., low concentrations prevented or reduced the increase in ROS formation, while high concentrations were pro-oxidant. In this sense, we emphasize that both doses used in this study can be considered antioxidants beneficial to the health of sheep concerning antioxidant/oxidant status.

The ewes fed with the phytogenic had lower SCCs. This can be related both to the decrease in neutrophil counts that occurred in the same period (anti-inflammatory action; Lima et al. 2013), as well as to the decrease in microorganisms (antimicrobial action; Chao et al. 2000) of the compound present in the blend and used here, improving the quality of the milk produced. Mastitis is one of the causes of substantial economic losses in dairy properties, as this pathology is characterized by inflammation of the udder, with high SCCs in milk as a result of microbial infection (Alba et al. 2019). The antimicrobial actions of thymol and carvacrol are related to their phenolic compositions, which makes them hydrophobic and capable of increasing the permeability of cell membranes and even their rupture, causing extravasation of the cellular contents and death of microorganisms (Alagawany et al. 2015, Benchaar et al. 2008). It is also reported that gram-positive bacteria are more susceptible to these compounds when compared to gram-negative bacteria that have an extra layer around their cell membrane; however, compounds such as thymol and carvacrol can also act on it by virtue of their low molecular weights (Benchaar et al. 2008).

Cholesterol showed higher values than the reference for sheep (52–76 mg/dL, Meyer & Harvey 2004) in all treatments, similar results from other studies that reported a physiological increase in cholesterol in ruminants as lactation days progressed (Ruas et al. 2000, Godoy et al. 2004). Nevertheless, the highest value was

in the group treated with the lowest doses of phytogenic, the same group with the highest milk production in the final days of our study. High cholesterol levels can also be explained during lactation due to increased plasma lipoprotein synthesis or periods of fasting due to the mobilization of body fat (Gonzáles & Silva 2008).

The results of our work revealed an increase in total proteins in the animals that received the additive due to the increase in globulins since the albumin values did not change. The sum of albumin and globulins forms total serum proteins. By subtracting the value of albumin from the total protein, we obtain the value of globulins (Meyer & Harvey 2004). Globulins function in plasma transport of metals, lipids, and bilirubin, in addition to actively participating in immune responses (Gonzáles & Silva 2008). Immunoglobulins protect against pathogenic microorganisms, preventing damage to cell surfaces (Namkung et al. 2004). Hyperglobulinemia may be associated with increased immunoglobulin synthesis, just as it occurs in a vaccine immune response (Jarikre et al. 2019) or after transferring colostral immunity protecting against infections (Heradéz-Castellano et al. 2014). Thus, the increase in plasma globulin levels can be associated with an increase in the immune response of supplemented animals.

Greater GGT activity of animals that received the phytogenic may be due to increased liver activity in sheep that received the additive. A similar result was reported by Castillo et al. (2012) in cattle supplemented with carvacrol and cinnamaldehyde. GGT is present in cell membranes in various tissues, particularly renal tubular cells and bile duct epithelium (Franciscato et al. 2006); increases in its activity can be a consequence of the increase in the concentration of bile acids or cholestasis (Kerr 2003, Gonzáles & Silva 2008), which can be correlated to the increase in cholesterol we found in this study.

CONCLUSION

A blend containing 21.55 mg of carvacrol, 18.76 mg of thymol, and 27.62 mg of cinnamaldehyde per gram of phytogenic agent, added to sheep feed after the peak of lactation increased production efficiency and reduced feed conversion. The consumption of the additive by the sheep stimulated humoral immune responses, increasing levels of globulins, reducing neutrophil counts and serum ROS, and primarily reducing the count of somatic cells in milk. In general, the additive used in the sheep's diet improves milk production and quality.

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