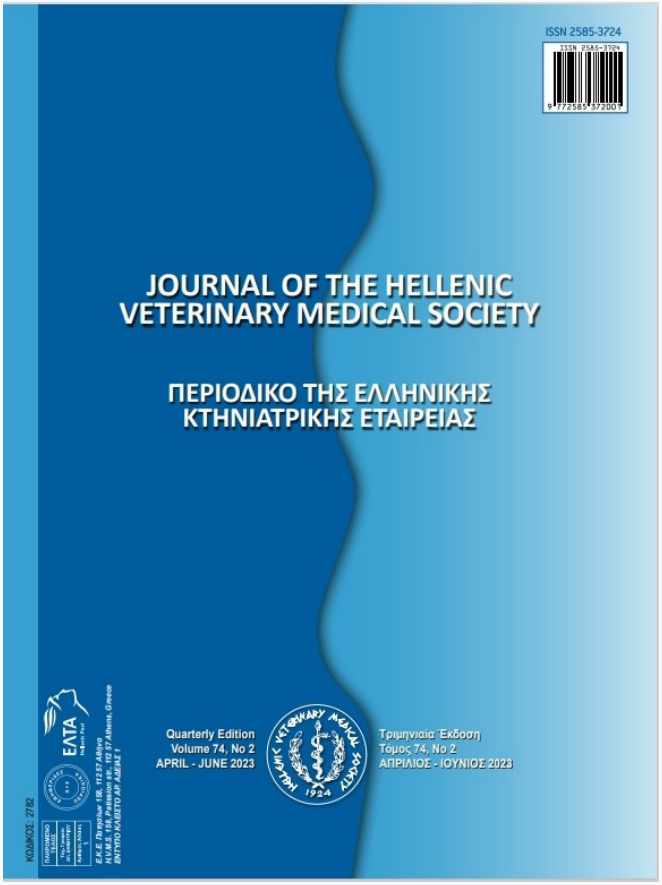
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Research article
Ερευνητικό άρθρο

Effects of rosemary essential oil as a feed additive on performance, rumen fermentation, and blood parameters in preweaning Holstein calves

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ABSTRACT: The objective of this study was to investigate the effects of rosemary essential oil (REO) supplementation on growth performance, blood metabolites and rumen fermentation in calves throughout the suckling period. Fourty Holstein calves were randomly divided to four dietary groups. Each group consisted of 10 calves; control with no REO supplementation (CON), supplementation of 500 mg/d REO (REO1), supplementation of 1000 mg/d REO (REO2) and supplementation of 2000 mg/d REO (REO3). REO supplementation quadratically increased ($P<0.05$) the calf starter (CS) intake, average daily gain (ADG) and feed efficiency. Calves fed REO1 and REO2 had the highest CS intake and ADG. Ruminal ammonia-N concentration was lower ($P=0.02$) for calves fed REO3 than calves fed REO1, but total volatile fatty acids concentration was higher ($P<0.01$) for calves fed REO1 compared with calves fed CON and REO3. The concentrations of ghrelin, NEFA and BHBA increased linearly ($P<0.05$) with increasing levels of REO. Calves fed REO2 and REO3 had the highest concentration of ghrelin. Cholesterol concentration decreased linearly ($P<0.01$) with increasing REO levels on d 56. Calves fed REO2 and REO3 had the lowest cholesterol concentration. Also, serum IgG concentration was higher ($P<0.01$) in calves fed REO2 and REO3 compared with calves fed CON on d 28. It was concluded that the addition of different amounts of rosemary essential oil can positively change some rumen and blood metabolites of calves, as well as the supplementation of REO may have a beneficial effect on growth performance by increasing ghrelin.

Keywords: calf; essential oil; ghrelin; growth performance; rosemary

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INTRODUCTION

As antibiotic resistance has become a public health concern, the use of antibiotics as growth promoters for livestock has been extensively banned in many countries (Benchaar et al., 2008). Newborn calves need antibiotic alternatives due to stress and diseases that can cause high morbidity and mortality (NAHMS, 2016). Therefore, the use of various alternate feed additives has been investigated to heal performance and overall health (Kertz et al., 2017). Essential oils (EO) are produced from plants that can be utilized as natural alternatives to in-feed antibiotics (Biricik, 2016). The use of essential oils in calves has significant benefits on performance and health parameters. Hill et al. (2007) reported improved ADG, CS intake, and feed efficiency in growing neonatal calves fed EO blend. However, results regarding the impacts of EO on performance have not been consistent, most likely due to variations in the chemical structure and dosage of the EO used (Schären et al., 2017). For example, Campolina et al. (2021) reported that the EO blend had no benefits on the BW gain, CS intake and feed efficiency in calves. Essential oils can cause disruption of membrane structure and inactivation of microbial enzymes due to disruption of protein translocation, phosphorylation and enzyme-related reactions of ruminal microorganisms (Benchaar et al., 2008). In general, essential oils have been shown to reduce methane production by the rumen archaea by causing changes in archaeal populations or the activity of methanogens (Ohene-Adjei et al., 2008). However, it has been stated that high concentrations of essential oil can cause a general inhibition of rumen fermentation (Bodas et al., 2012). Moreover, Katsoulos et al. (2017) showed that essential oils can reduce the severity and incidence of diarrhea in calves. The knowledge from these studies are generally improbable and the variability of dosages, chemical nature of EO compounds and blends can be cited as the reason for these different results (Calsamiglia et al., 2007).

Many EO and their components have been studied on calves as an alternative to antibiotics. However, it is surprising that no studies have been specifically designed to prove rosemary essential oil (REO) on calf performance and health. Rosemary is a natural source of polyphenols that can be used in the diets of animals. Rosemary contains phenolic diterpenes such as rosmanol, carnasol, carnosoic acid, 7-methyl-epirosmanol and isorosmanol, and phenolic acids such as caffeic and rosmarinic acids. In addition, monoterpenes such as 1,8-cineol, α-pinene, β-pinene, borneol and camphor are the main components of REO. These compounds have strong antibacterial and antimicrobial influences against some fungi and pathogenic bacteria (Okoh et al., 2010). Rosemary is known to lower serum triglyceride and total cholesterol, as well as increase the humoral immune response (Ghozlan et al., 2017; El-Gogary et al., 2020). It is stated that rosemary has the ability to reduce rumen ammonia nitrogen in ruminants due to the phenolic diterpenes it contains (Sahraei et al., 2014).

Rosemary has been used in human nutrition for many years to change the taste of food and to increase appetite. Ghrelin has been found to stimulate appetite, play an important role in energy and glucose homeostasis, and stimulate growth hormone (Zhang et al., 2008). Studies in beef cattle have shown that exogenous active ghrelin can increase time spent eating and DMI (Wertz-Lutz et al., 2006). Foote et al. (2014) showed that active ghrelin is positively associated with DMI in finishing cattle. However, the effect of REO on the ghrelin hormone is still unknown. In addition, there is insufficient information on the effects of rosemary essential oil on performance, blood and rumen parameters in dairy calves. Our hypothesis is that the addition of REO to calves can be used to improves rumen development, blood metabolites and immunity, as well as increasing the growth performance of calves by stimulating ghrelin. The objective was to determine if an inclusion of REO in the milk replacer (MR) could improve calf performance with fewer health challenges in Holstein dairy calves.

MATERIALS AND METHODS

Ethical approval

The experiment was conducted under an approved protocol by Animal Care and Use Committee of University of Uludag (protocol ID: B.30.2.ULU.0.8Z.00.00/14).

Animal and experimental design

This experiment was conducted at the Omer Matli Academy Livestock Research and Development Center in Bursa located within the North West Turkey. Forty Holstein calves in the study were procured from the commercial research dairy farm, Omer Matli Farm (24 male and 16 female calves equally divided between treatments). The calves on the farm were housed in individual sheds with straw sills and natural ventilation (between 1 and 56 d of age). At birth (d 1), blood samples were collected from calves for

evaluation of total serum protein (TSP). All calves received 4 L colostrum up to 6 h after birth. Calves at 2 to 3 days of age were fed 5 L/d of colostrum divided into two equal meals served at 0900 and 1700 h. At 4 d of age, calves were fed daily 5 L of MR (Trouw Nutrition Inc., Amersfoort, Netherlands), divided into two equal meals until 49 d of life (125 g of MR powder in per L). At 49 d of life, MR was fed only in the mornings and at 56 d of life, calves were weaned. Water and CS (Matlı Feed Industry, Karacabey, Turkey) were given *ad libitum* and 100 g of alfalfa hay from 4 d of age. The ingredient formulation of CS was performed according to the NRC (2001) nutrient requirements for calves throughout the first 56 d of life (Table 1). The nutrient composition of CS, MR and alfalfa are shown in Table 2.

Table 1. Ingredient composition of calf starter

Ingredients	% of mix (DM* basis)
Corn, ground	35.0
Wheat bran	14.5
Molasses, beet sugar	5.0
Corn dry distiller grain with soluble	11.0
Soybean meal (48 % CP†)	12.0
Canola meal	11.0
Corn gluten feed	9.0
Limestone	1.6
Salt	0.80
Vitamin and trace minerals ††	0.10

* DM: dry matter.
† CP: crude protein.
†† Contained 20000000 IU of vitamin A/kg, 3000000 IU of vitamin D/kg, and 25000 mg of vitamin E/kg, 4000 mg of vitamin B_1/kg, 8000 mg of vitamin B_2/kg, 5000 mg of vitamin B_6/kg, 20 mg of vitamin B_{12}/kg, 20000 mg of niacin/kg, 200000 mg of choline chloride/kg, 50000 mg of Mn/kg, 50000 mg of Fe/kg, 50000 mg of Zn/kg, 10000 mg of Cu/kg, 800 mg of I/kg, 150 mg of Co/kg, 150 mg of Se/kg.

Calves (4 d of age) were randomly divided into four groups (10 animals each) with similar mean body weight (43.07 ± 2.6 kg of BW), and total serum protein. The groups were prepared as follows: control with no REO supplementation (CON); supplementation of 500 mg/d REO in the milk replacer (REO1), supplementation of 1000 mg/d REO in the milk replacer (REO2) and supplementation of 2000 mg/d REO in the milk replacer (REO3). From d 4 to weaning, determined amounts of REO were added to MR of each calf at 0900 h morning feed. ,

Rosmarinus officinalis leaves were collected from Mersin, Turkey in June. REO was obtained by steam distillation from the leaves, which were dried and passed through a 2 mm sieve.

Determination of essential oil components in rosemary

Hydrodistillation method used for extraction of bioactive compounds of rosemary according to the method recommended in the Turkish Standards (TS ISO 356). Gas chromatography analysis was performed on an MS-Thermo Polaris Q GC-Thermo Trace GC (Thermo Ficher inc, MA, USA) ultra equipped with a fused HP5-MS capillary column (Thermo Ficher inc, MA, USA) (30x 0.25x film thickness 0.5 µm). The temperature is programmed to rise from 60°C to 240°C at 4°C/min. Injection was carried out in split mode at 250°C. Helium gas at 1.3610 atm was used as the carrier. The injection volume was 0.1 µL for all samples and detection was performed by FID at 250°C. Chromatograms were determined using mass spectrometry. Data were calculated using inner standards (Pala-Paul et al., 2004).

Table 2. Nutrient compositions of calf starter, alfalfa hay and milk replacer on a dry matter (DM) basis

Nutrients	Calf starter	Alfalfa hay	Milk replacer
DM (%)	89	92.38	96.5
Crude Protein (%)	22	16.43	21
Ether extract (%)	4.2	1.8	16.5
Crude ash (%)	6.71	12.37	7.4
NDF* (%)	23.1	57.14	0.1
ADF† (%)	9.9	48.72	0.0
NFC†† (%)	46	11.03	55
Ca (%)	0.88	1.38	0.77
P (%)	0.67	0.25	0.55

* NDF : neutral detergent fibre
† ADF : acid detergent fibre
†† NFC (non-fibre carbohydrate): 100- (% CP + % NDF + % Ash + % Ether extract).

Feed intake and analysis

The amounts of MR, CS and alfalfa hay were weighed individually and offered to the calves from d 4. The next day, the refusal feed from the intake of the calves was collected, and the daily consumed CS and DM amounts were calculated. CS and alfalfa hay samples were collected weekly and combined in monthly batches for nutrient analysis at the end of the study. All feed samples were dried in air-forced at 55 °C (WTB BINDER, Germany) for 72 h and ground through a 1 mm sieve (Retsch Cross Beater Mill SK 100, Germany). MR, CS, and alfalfa hay were analyzed to determine DM, CP, crude ash, ether extract (EE) according to AOAC (2) (Table 2). NDF and ADF were determined using the method defined by Van Soest et al. (1991). DM intake was calculated daily DM content of MR, CS and alfalfa hay.

Body weight, rumen and health measurements

In order to determine the average daily live weight gain, the calves were weighed weekly during the experimental period and the weight gain was divided by the number of days. In addition, feed efficiency (ADG/kg total daily DMI) was calculated for each calf.

On d 56 of the experiment, rumen samples were gathered by gastric tube approximately 4 h after the morning feeding. A speculum was placed in the mouth, and a lubricated rubber tube was entered into the rumen through the esophagus. Ruminal contents (25-50 mL) were taken using an electric pump, and the part contaminated with saliva was removed. Then, ruminal pH was immediately measured using a pH meter. (Inolab pH, serial no: 00200018, pH Electrode SenTix 41, D-82362, Weilheim, Germany). In addition, rumen fluid was used to determine the concentration of ammonia-N and VFA. Rumen fluids were filtered using 4 layers of cheesecloth with a mesh size of 250 µm. For VFA analysis, samples were acidified using 2 mL of 25% metaphosphoric acid. To determine the ruminal ammonia-N concentration, 5 ml of filtrate sample was preserved by adding 1 ml of 1% H_2SO_4. After the samples were centrifuged at 5000 rpm for 10 min, the supernatants were stored frozen at -20°C until analysis. Ruminal VFA concentrations were determined using gas chromatography (Hewlett Packard Agilent Technologies 6890N Network GC System, Serial CN10447002, Beijing, China). A column (6 × 2 mm ID glass) was packed with 10% SP-1200/1% H_3PO_4 80/100 Chromosorb WAW (Supelco, Bellefonte, PA, USA). Carrier gas (N2) flow was 30 mL/min, inlet temperature was 175°C, detector temperature was 170°C, and column temperature was 110°C. Detection was carried out by flame ionization. Ruminal ammonia-N concentration was determined using the method described by Annino (1964).

On d 28 and 56, blood samples were collected from the jugular vein of each calf 3 h after morning feeding. Samples were collected in a 10-mL vacuum tube of both nonheparinized and K3EDTA to evaluate serum and plasma. After the blood tubes were placed on ice, they were centrifuged at 4000 rpm for 10 min at 4°C. Plasma and serum samples were stored at -20°C until analysis. The blood glucose, triglyceride, total cholesterol, and total protein concentrations in each serum samples were determined using spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, JAPAN) with a commercial diagnostic kits (Ben S.r.l-Biochemical Enterprise, Milano, ITA). Levels of β-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA), immunoglobulin G (IgG), ghrelin concentrations were measured using commercial ELISA test kits (MyBioSource, Inc., San Diego, CA, USA) with Biotek Elisa Reader (BioTek Instruments, VT 05404-0998, Winooski-USA).

Statistical analysis

All data were analyzed for normality with Shapiro-Wilk test and homogeneity were evaluated using Levene test. Data on intakes, BW, ADG, feed efficiency and blood metabolites excluding rumen parameters were analyzed by a repeated measurement procedure using the General Linear Model (GLM). The analysis includes the main effect of treatment between subjects, the main effect of sampling time within subjects, and the interaction between sampling times × treatment. In this case, all data were analyzed by one-way ANOVA analysis of variance with Bonferroni correction. Polynomial contrasts (linear and quadratic) were used to determine the effects of REO levels on measured variables with the control. Differences between means were defined using Tukey multiple comparisons. The differences were considered significant at $P \leq 0.05$. Statistical analyses were performed by using SPSS software version 23.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Chemical composition of rosemary essential oil

The main components of rosemary essential oil are 1,8-cineol (51.63%), α-pinene (11.77%), cam-

phene (5.21%), borneol (4.16%) and β-caryophyllene (3.78%) were determined as (Table 3).

Growth performance

Results showed that increasing levels of REO supplementation (P<0.05) quadrically increased total DM and CS intake. CS intake was higher (P<0.001) for calves fed REO1 and REO2 compared to calves fed CON and REO3 (Table 4). Total BW, ADG and feed efficiency increased quadratically (P<0.05) with increasing levels of REO and calves fed REO1 and REO2 had the highest (P<0.001) total BW gain and ADG.

Table 3. Chemical composition of rosemary essential oil (*Rosmarinus officinalis*)

Components	(%)
α-pinene	11.77
Camphene	5.21
β-pinene	1.29
Sabinene	0.54
β-myrcene	0.85
Limonene	1.89
1,8-cineol	51.63
p-cymene	3.12
Camphor	3.11
Linalool	0.63
Bornyl acetate	2.56
β-caryophyllene	3.78
α-terpineol	2.73
Borneol	4.16
Caryophyllene oxide	0.75

Rumen fermentation parameters

Ruminal pH numerically increased for calves fed REO1 and REO2, but there was no significant difference among groups (Table 5). Calves fed REO3 was lower (P=0.02) ruminal ammonia-N concentration than calves fed the REO1. The concentration of total VFA was decreased (P<0.05) for calves fed REO3 compared with calves fed REO1 and REO2. There was a quadratic increase (P<0.05) in propionate and acetate with REO supplementation. The concentrations propionate and acetate were higher (P<0.001) for calves fed REO1 compared with calves fed REO3 and CON. Ruminal propionate concentration was lowest (P<0.01) for calves fed REO3. There was no difference among groups in terms of butyrate concentration. Calves fed REO3 had higher (P=0.03) valerate concentration than calves fed CON, but concentration of isovalerate was lower (P<0.001) for calves fed REO3 than other groups.

Blood parameters

Ghrelin concentration was affected on d 28 and 56 of the study and increased linearly (P<0.01) with increasing REO levels. Ghrelin concentration was lowest (P<0.001) in calves fed CON on both sampling days (Table 6). The concentrations of serum NEFA and BHBA increased linearly (P<0.05) with increasing levels of REO. Serum NEFA levels were higher (P<0.001) for calves fed REO3 on d 28 and 56 compared with the other groups. Serum BHBA levels were higher (P<0.05) in all groups in which REO was supplemented on d 28 and 56 compared with calves

Table 4. Average total DMI, dry matter intake of CS, dry matter intake of alfalfa, BW, ADG and feed efficiency (G:F) of calves fed milk replacer containing rosemary essential oil

	Treatments*					*P-values*		
Item	CON	REO1	REO2	REO3	SEM	Treatment	Linear	Quadratic
Initial BW (kg)	43.59	42.45	42.26	44.02	0.97	0.63	0.86	0.26
Final BW (kg)	62.92^{b}	68.00^{a}	67.83^{a}	66.62^{a}	0.61	<0.001	0.38	0.13
Total gain (kg)	19.33^{c}	25.55^{a}	25.57^{a}	22.60^{b}	0.77	<0.001	0.19	0.02
ADG† (kg/d)	0.342^{c}	0.456^{a}	0.456^{a}	0.403^{b}	0.01	<0.001	0.20	0.02
Calf Starter DMI (kg/d)	0.357^{b}	0.497^{a}	0.501^{a}	0.414^{b}	0.01	<0.001	0.43	0.03
Alfalfa hay DMI (kg/d)	0.024^{c}	0.034^{b}	0.036^{b}	0.039^{a}	0.01	<0.001	0.19	0.45
Total DMI†† (kg/d)	0.840^{b}	0.972^{a}	0.979^{a}	0.901^{ba}	0.02	0.02	0.34	0.04
Feed efficiency^{ϕ}	0.40^{b}	0.47^{a}	0.47^{a}	0.44^{a}	0.01	<0.001	0.06	<0.01

*CON: control, ROE1: supplementation of 500 mg/d rosemary essential oils in the milk replacer, ROE2: supplementation of 1000 mg/d rosemary essential oils in the milk replacer, ROE3: supplementation of 2000 mg/d rosemary essential oils in the milk replacer.
† ADG: average daily gain.
†† Total DMI: total dry matter intake (milk replacer plus calf starter plus alfalfa hay).
^{ϕ} ADG/kg total daily DMI.
$^{a-c}$Means within a row with different superscripts differ (P<0.05).

Table 5. Mean values of ruminal pH, ammonia nitrogen (NH_3-N) and volatile fatty acids (VFA) of calves fed milk replacer containing rosemary essential oil

	Treatments*					*P- values*		
Item	**CON**	**REO1**	**REO2**	**REO3**	**SEM**	**Treatment**	**Linear**	**Quadratic**
pH	5.64	5.90	5.74	5.60	0.13	0.30	0.61	0.10
NH_3-N (mmol/L)	1.52^{ba}	1.62^{a}	1.54^{ba}	1.40^{b}	0.06	0.04	0.94	0.30
Total VFA(mmol/L)	116.05^{b}	132.08^{a}	122.23^{ba}	108.11^{bc}	3.00	<0.01	0.46	0.15
Acetate	47.33^{b}	56.24^{a}	49.40^{bc}	41.93^{bd}	1.49	<0.001	0.18	0.04
Butyrate	16.24	16.66	14.17	17.28	1.10	0.22	0.92	0.52
Propionate	31.56^{b}	36.15^{a}	36.32^{a}	24.81^{c}	1.14	<0.001	0.27	0.04
Valerate	18.40^{b}	20.39^{ba}	19.69^{ba}	22.40^{a}	0.89	0.03	0.37	0.88
Isovalerate	2.31^{a}	2.09^{a}	2.29^{a}	1.56^{b}	0.06	<0.001	0.06	0.30

* CON: control, ROE1: supplementation of 500 mg/d rosemary essential oils in the milk replacer, ROE2: supplementation of 1000 mg/d rosemary essential oils in the milk replacer, ROE3: supplementation of 2000 mg/d rosemary essential oils in the milk replacer.
[a-d]Means within a row with different superscripts differ (P<0.05).

Table 6. Mean values of blood metabolites of calves fed milk replacer containing rosemary essential oil

	Treatments*					*P- values*		
Item	**CON**	**REO1**	**REO2**	**REO3**	**SEM**	**Treatment**	**Linear**	**Quadratic**
Ghrelin (ng/mL)								
d 28	1039.54^{b}	1013.09^{b}	1243.33^{a}	1280.80^{a}	22.79	<0.001	<0.01	0.31
d 56	973.72^{c}	1080.18^{b}	1192.33^{a}	1199.00^{a}	16.79	<0.001	<0.01	0.46
NEFA (mEq/mL)								
d 28	73.72^{b}	76.54^{b}	78.20^{b}	91.20^{a}	1.30	<0.001	<0.001	0.10
d 56	75.36^{b}	79.54^{b}	71.10^{b}	93.30^{a}	1.80	<0.001	0.02	0.09
BHBA (mmol/L)								
d 28	0.835^{c}	1.032^{b}	1.122^{ba}	1.154^{a}	0.02	<0.001	<0.001	0.18
d 56	0.878^{d}	1.105^{b}	0.959^{c}	1.226^{a}	0.02	<0.001	<0.01	0.48
Glucose (mg/dL)								
d 28	106.09	99.40	105.77	100.60	2.51	0.29	0.64	0.78
d 56	93.00	95.60	96.55	98.10	2.22	0.60	0.55	0.98
Total cholesterol (mg/dL)								
d 28	106.81^{b}	118.09^{a}	118.20^{a}	100.90^{b}	2.24	<0.001	0.32	0.06
d 56	130.54^{a}	127.27^{a}	113.30^{b}	102.20^{b}	2.80	<0.001	<0.01	0.68
Triglycerides (mg/dL)								
d 28	52.63^{ba}	56.73^{ba}	59.30^{a}	50.40^{b}	1.52	<0.001	0.28	0.02
d 56	59.91^{b}	63.63^{ba}	57.20^{b}	69.29^{a}	1.68	<0.01	0.27	0.09
Total protein (g/dL)								
d 28	7.16^{a}	5.15^{c}	5.96^{b}	5.53^{cb}	0.19	<0.001	<0.01	<0.001
d 56	6.15	5.43	5.89	5.59	0.16	0.06	0.02	0.25
IgG (g/dL)								
d 28	6.70^{b}	7.17^{ba}	8.01^{a}	8.05^{a}	0.27	<0.01	<0.01	0.54
d 56	6.90^{b}	7.48^{ba}	7.63^{ba}	8.10^{a}	0.22	0.01	<0.001	0.86

* CON: control, ROE1: supplementation of 500 mg/d rosemary essential oils in the milk replacer, ROE2: supplementation of 1000 mg/d rosemary essential oils in the milk replacer, ROE3: supplementation of 2000 mg/d rosemary essential oils in the milk replacer.
[a-d]Means within a row with different superscripts differ (P<0.05).

fed CON. Glucose concentration was not affected by REO supplementation. Serum cholesterol concentration increased (P<0.001) for calves fed REO1 and REO2 compared with calves fed CON and REO3 on 28th d. However, on d 56, cholesterol concentration decreased linearly (P<0.01) with increasing levels of REO and calves fed calves fed REO2 and REO3 had lowest (P<0.001) cholesterol concentration. At d 28 of the study, serum triglyceride and total protein concentrations decreased quadratically (P<0.01) with increasing levels of REO supplementation. Serum triglyceride concentration was lower (P<0.001) for

calves fed REO3 compared with the calves fed REO2 on d 28. However, the concentration of triglyceride increased (P<0.01) in calves fed REO3 and was higher than calves fed CON and REO2 at the end of the study. On d 28 and 56 of the study, serum IgG concentration increased linearly (P<0.01) with REO supplementation and the highest value was determined in calves fed REO3. In addition, IgG concentration was higher (P<0.01) for calves fed REO2 compared with the calves fed the CON on d 28.

DISCUSSION

In the current study observed that 1,8-cineol (51.63%) constituted the major part of the essential oil obtained from rosemary, and also α-pinene, camphene, borneol and β-caryophyllene as minor components. Similarly, Mathlouthi et al. (2012) stated that the main active ingredient of rosemary is 1,8-cineol and it constitutes 49.9% of the essential oil. Other components were found to be α-pinene (9.95%), β-pinene (6.53), caryophyllene (3.89%), camphene (3.66%) and borneol (3.45%).

The results of this study showed that the use of REO increased DM intake. This finding can be concluded that phenolic compounds found in medicinal plants can increase appetite in animals. Some researchers have determined the digestibility of NDF, ADF, Cellulose, and hemicellulose with REO addition (Kholif et al. 2017, Smeti et al.2015). In this study, there was an increase in roughage consumption due to the addition of REO Similarly, Jeshari et al. (2016) reported that EO blend (300 mg/kg feed) including REO increased the CS and total DM intake in calves. However, Campolina et al. (2021) reported that EO blend including REO was not effective on the starter intake, total DM intake, BW gain and feed efficiency in calves.

The greater growth rate observed in calves fed REO may be due to higher CS and total DM intake, while overdose REO3 (2000 mg/d) supplementation may reduce the increase in calves performance. The use of high doses of EO may cause a decrease in enzyme activity, and also cause intestinal problems by creating changes in the anatomy and physiology of the intestinal epithelium (Durmic and Blache, 2012). According to other studies, EOs stimulated feed intake and the secretion of digestive enzymes, improved the digestion of food and the rate of passage through the intestines and increased growth performance (Jamroz and Kamel, 2002; Bento et al., 2013). EOs stabilize the gastrointestinal system by controlling the growth of potentially pathogenic microorganisms in the gastrointestinal tract (Castillo et al., 2006). It has been reported that animals are less exposed to microbial toxins, ammonia and biogenic amines with the addition of EOs (Windisch et al., 2008). In addition, EOs have been shown to increase the intestinal mucosa's absorption capacity of nutrients by increasing the depth of the crypt and the length of the intestinal villi (Jamroz et al., 2006). Similar to this study, Froehlich et al. (2017) observed that the addition of 1.25 g/d EO blend into the MR increased the growth performance in calves. All these findings demonstrate the beneficial effects of EOs on growth performance. However, Shokrollahi et al. (2015) reported that the use of different doses of REO did not have a positive effect on BW gain performance on goat kids.

In the current study, supplementation of different doses of REO did not affect the rumen pH of calves. Similarly, Castillejos et al. (2008) reported in an in vitro study that the use of rosemary oil at different doses did not affect ruminal pH. However, Salem et al. (2013) reported that polyphenols increase the amount of saliva and can increase ruminal pH due to the buffering effect of saliva.

According to the current results, calves fed REO3 decreased ruminal ammonia-N. The current observation is in line with the findings of Sahraei et al. (2014) who observed that the use of 400 mg/d REO reduces ammonia-N in sheep. This effect may be due to the fact that phenolic compounds reduce the number and diversity of ammonia-producing bacteria (Wanapat et al., 2013). The suppressive effects of phenolic compounds on ruminal ammonia-N may be due to a decrease in the rate of fractional degradation or decrease the immediately degradable fraction of the protein (Frutos et al., 2004). It may be more beneficial for amino acids to be absorbed from the small intestines by preventing their degradation in the rumen (Wallace, 2004). The effects of REO3 supplementation in this study may have resulted from the effect of 1,8 cineole, as it was the main active ingredient. In contrast, Santos et al. (2015) reported that the addition of EO blend into the MR and CS increased the concentration of ammonia-N in calves. Accordingly, they stated that essential oils did not modulate deamination and did not affect ammonia-producing bacteria. Similarly, Castillejos et al. (2008) found that the use of REO in different doses did not affect concentration of ammonia-N in vitro study.

Since the CS intake was higher in the REO1 group, total VFA, acetate and propionate concentrations increased, indicating enhanced ruminal development. Similar to this finding, Poudel et al. (2019) stated that the use of EO significantly increased the concentration of ruminal propionate in calves and tended to increase in acetate and total VFA. However, Cobellis et al. (2015) reported that the addition of 1.75% REO to the ration did not change the total concentration and composition of ruminal VFA in rumen cannulated sheep. Calves fed REO3, concentration of total VFA, acetate and propionate tended to decrease compared with others. Similarly, Odhaib et al. (2018) reported that the use of rosemary leaves in the ration of Dorper lambs decreased the concentration of acetate, propionate and total VFA, but increased the concentration of butyrate. The decrease in the concentration of total VFA in calves fed REO3 may be due to the antimicrobial effect of the excess phenolic compounds (Odhaib et al., 2018). Pintore et al. (2002) stated that REO had more antimicrobial effect against gram-positive bacteria than negatives and decreased acetate and increased the concentration of propionate. These data demonstrated that different doses of REO changed rumen parameters and the best effect was found in the calves fed REO1.

Current results revealed that plasma ghrelin concentration increased according as the dose of REO increase. Ghrelin is a "hunger hormone" and at the same time, this hormone stimulates the secretion of growth hormone. As the dose of REO increases, the appetite increases proportionally and means that the growth hormone will be stimulated more (Hosoda and Kangawa, 2008). In our study, plasma ghrelin concentration increased according to the dose of REO, this situation is also observed in serum NEFA and BHBA concentrations. The increase of these negative energy balance markers relative to the dose of REO suggests that ghrelin is proportional to the induction of appetite or even growth. At the same time, the increase in BHBA concentration in the blood showed that the addition of rosemary essential oil had a positive effect on the development of the rumen. Chiofalo et al. (2012) found that the use of rosemary extract 600 mg/d in sheep decreased the level of NEFA, but increased the concentrations of BHBA and glucose. However, Campolina et al. (2021) stated that EO blend did not affect blood BHBA and glucose concentrations in calves.

At the end of the study, 1000 and 2000 mg REO supplementation daily reduced serum cholesterol. Hepatic 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase is the key enzyme in cholesterol synthesis, and phenolic substances can inhibit the activity of this enzyme (Crowell, 1999). However, El-Gogary et al. (2018) showed that using different doses of REO in rabbits did not change blood cholesterol and triglyceride concentrations. In this study, 2000 mg/d REO supplementation increased serum triglyceride at the weaning. Similar to this finding, Polat et al. (2011) stated that REO increased the triglyceride concentration in broilers. In contrast, Ghozlan et al. (2017) determinated that the use of rosemary in broiler diet reduces the amounts of serum triglyceride and total cholesterol.

In accordance with the present finding, REO supplementation numerically reduced total serum protein concentration in the first 4 wk, but this difference was not observed at the end of the study. Similarly, Odhaib et al. (2018) reported that the addition of rosemary leaves to the ration of Dorper lambs did not change total serum proteins. However, Chiofalo et al. (2012) stated that the use of different doses (600, 1200 mg/d) of REO in sheep linearly increased the total protein concentration.

Current study showed that REO positively affects IgG. The reason for this increase in IgG concentration may be due to B lymphocyte stimulation (Hou et al., 2007). With the increase of immunoglobulins, the immune response begins with helper T cells, cytotoxic T cells and C-T cells (Wright et al., 1994). In agreement with these data, El-Gogary et al. (2020) reported that the use of 1g/kg rosemary extract in broiler chick diets increased the concentrations of IgG, IgM and IgA as a humoral immune response. In addition, Liu et al. (2020) showed that the EO blend (44.1 mg/kg starter) containing the active ingredients of REO increased IgG, IgA and IgM concentrations in calves. However, Froehlich et al. (2017) stated that the EO blend in different doses, including the active ingredients of rosemary, did not change the IgG and IgA concentrations in calves.

CONCLUSION

The results of this study showed that daily supplementation of rosemary essential oil to calves can improve feed intake and growth performance by increasing ghrelin concentration. In addition, supplementation of 500 mg of rosemary essential oil increased ruminal total volatile fatty acids by promot-

ing ruminal fermentation in calves. Moreover, supplementation of 1000 and 2000 mg of rosemary essential oil enhanced the immune response by increasing IgG in calves. Taken together, rosemary essential oil can be used as an alternative additive to heal calf health and performance.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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