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Effects of emulsified essential oils blend on performance, blood metabolites, oxidative status and intestinal microflora of suckling calves

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ABSTRACT

A blend of essential oils (EOs) from four medicinal plants (*Thymus kotschyanus, Lavandula angustifolia, Salvia officinalis* and *Capparis spinosa*) were emulsified in water and dissolved in the daily milk allotment of calves to evaluate their effects on growth performance, blood metabolites, oxidative status, and intestinal microflora. Forty 7-day old male Holstein calves were randomly assigned to four dietary treatments (n = 10 calves/group) in a completely randomized design. Treatments were: 1) control group received no EOs (CON), 2) calves received 100 mg of a blend of EOs/d by including 2 ml of the emulsion medium in milk (100E), 3) calves received 200 mg of a blend of EOs/d by including 4 ml of the emulsion medium in milk (200E), and 4) calves received 300 mg of a blend of EOs/d by including 6 ml of the emulsion medium in milk (300E). Data were analyzed as a completely randomized design with repeated measurements in time by using the PROC MIXED of SAS. Feeding calves with a blend of EOs in emulsified form resulted in a linear increase in average daily gain during 14–28 d ($P = 0.02$) and 28–42 d ($P = 0.01$). Similarly, increasing doses of the EOs resulted in a linear increase in triglyceride concentration on d 15 ($P = 0.01$) and d 30 ($P = 0.03$). On the contrary, blood urea concentration decreased linearly ($P = 0.01$) on d 15 with increasing doses of the emulsified EOs. Blood level of gamma glutamyl transferase decreased linearly ($P = 0.01$) on d 15 and 30 with EOs inclusion. Concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood were not affected by the treatments after 15 days of feeding the emulsified EOs. However, after 30 days, blood ALT ($P = 0.03$) and AST ($P = 0.01$) concentrations decreased linearly. Malon di-aldehyde concentration in blood decreased linearly with increasing doses of the emulsified blend of EOs ($P = 0.01$). Inclusion of EOs resulted in a linear increase ($P = 0.01$) in total antioxidant status on d 15 of the experiment. Additionally, feeding EOs in emulsified form resulted in a decrease in

Abbreviations: ADG, average daily gain; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; BHBA, beta-hydroxy butyric acid; BUN, blood urea nitrogen; EOs, essential oils; FCR, feed conversion ratio; GGT, gamma glutamyl transferase; GLM, general linear model; GPx, glutathione peroxidase activity; MDA, malondialdehyde; SOD, superoxide dismutases; TAS, total antioxidant status.

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fecal *E. coli* count ($P = 0.01$). Based on the results, feeding EOs in emulsified form could be an effective way to administer EOs to improve the health status of suckling calves.

1. Introduction

Early robust health program for calves will ensure strong and healthy calves at weaning. Calves are usually given colostrum at birth to gain passive immunity from maternal antibodies. However, environmental and production stressors may cause failure in passive transfer of maternal antibodies to the calves thereby making them susceptible to enteric and respiratory diseases (Hulbert and Moisa, 2016). To prevent or minimize these problems, antibiotics are added in the feed or in the milk or milk replacers (Salazar et al., 2019). However, due to documented evidence of antibiotic-resistant bacteria and their possible effects on human health, researchers are encouraged to find alternatives to antibiotics. Essential oils (EOs) from herbs can potentially replace antibiotics use in livestock production (Salazar et al., 2019). Several studies have reported positive effects of different EOs on the growth performance and health status of suckling calves (Santos et al., 2015; Jeshari et al., 2016; Hassan et al., 2020).

Thymus kotschyanus Bioss. is a native herbal plant of Iran belonging to the *Thymus* genus and has been widely used in traditional medicine based on its antibacterial, anti-inflammatory and antioxidant properties. The major components are carvacrol, thymol, pulegone, isomenthone, 1, 8-cineole, piperitenone and γ-terpinene (Rasooli and Mirmostafa, 2003). *Salvia officinalis* L. is native to the Middle East and Mediterranean areas. It has a wide range of pharmacological activities including anticancer, anti-inflammatory, antinociceptive, antioxidant, and antimicrobial effects (Bozin et al., 2007). *S. officinalis* contains different compounds such as borneol, camphor, caryophyllene, cineole, elemene, humulene, ledene, pinene, and thujone (Ghorbani and Esmaeilizadeh, 2017). *Lavandula angustifolia* is a perennial shrub from the Lamiacea family and its EO has antiseptic, anti-inflammatory, antinociceptive, antifungal and antibacterial properties (Cavanagh and Wilkinson, 2005). *Capparis spinosa* L. is a shrub from the Capparaceae family and the fruit and root have been used extensively in traditional medicine. The main compounds found in the essential oil of the fruit and root of *C. spinosa* are methyl, isopropyl and butyl isothiocyanates (Afsharypuor et al., 1998). Essential oils may be administrated to calves through their milk or milk replacer (Santos et al., 2015; Hassan et al., 2020) or in a starter feed mix (Jeshari et al., 2016; Salazar et al., 2019). Administration of drugs and feed additives via milk or milk replacer is better due to their ability to bypass the rumen and minimize possible adverse effects of the drugs or feed additives on the rumen microorganisms and vice versa. However, EOs are mostly hydrophobic or lipophilic compounds and therefore they are not soluble in milk which may result in an uneven mixture, poor stability, and low bioavailability. One possible solution may be using a specifically designed delivery system such as emulsion.

Emulsion based delivery systems have been used extensively to deliver lipophilic chemical compounds, such as functional lipids and phytochemicals in the food, medical, and pharmaceutical industries (McClements et al., 2007). Delivering bioactive lipophilic compounds as an emulsion has been reported to increase palatability, dispersion, uniform mixture, and absorption, resulting in increased bioactivity (McClements et al., 2007; Roohinejad et al., 2018). Basically, milk is an emulsion of oil in water along with other nutrients such as protein, carbohydrate, vitamins, and minerals. It is hypothesized that the use of emulsion-based system to deliver EOs (as emulsified EOs) by mixing them in milk will be advantageous for the dairy industry.

There are several articles on emulsifying EOs for use in the cosmetic, food, and pharmaceutical industries (Loeffler et al., 2014; Wu et al., 2014; Leon-Méndez et al., 2018). However, there is no report on emulsified EOs being an effective way of delivering EOs to calves. The present study was designed to evaluate the potential effects of administering EOs by using an emulsion-based delivery system in suckling calves. Therefore, the objective of this study was to investigate the effects of an emulsified blend of EOs on performance, blood metabolites, oxidative status, and intestinal microflora of suckling calves.

2. Materials and methods

2.1. Essential oils preparation and emulsification

Lecithin and whey protein were purchased from a local market (Ehsan confectionary shop, Ardabil, Iran), gum Arabic from Sigma-Aldrich (Sigma-Aldrich, CAS Number, 9000-01-5), and ethanol (0.99 purity) from the Merck (Merck Millipore, CAS Number, 64-17-5). The medicinal herbs (*T. kotschyanus, L. angustifolia, S. officinalis* and *C. spinosa*) were harvested from the experimental farm of the Ardabil Agricultural Research Center (Ardabil, Ardabil province, Iran) and dried under shade for 2–3 days and used for EOs preparation. These herbs were selected based on their antimicrobial and antioxidant effects as mentioned above. A blend of the herbs at a ratio of 1:1:1:1 (dry weight) was mixed and their EOs was extracted by hydrodistillation method using a semi-industrial apparatus (Karami essential oils and distillates extraction manufactory).

To prepare the emulsified EOs, an oil-in-water emulsion was prepared by using olive oil as the lipid phase, lecithin, and gum Arabic as emulsifiers and whey protein as emulsion stabilizer (Traynor et al., 2013; Wu et al., 2014; Caporaso et al., 2016). Briefly, 25 g of lecithin was dissolved in 250 ml of olive oil by heating to 60 °C and mixed by using a magnetic stirrer for 10 min to prepare the lipid phase. An aqueous phase was prepared by dissolving 2 g of gum Arabic and 5 g of whey protein in 250 ml of previously heated distilled water (60 °C) by gentle mixing using a magnetic stirrer on a laboratory hot plate for 1 h (Ibanoglu, 2002). Thereafter, the lipid phase and the aqueous phase were mixed with each other in a kitchen blender for 1 h. Thereafter, 50 g of the blend of EOs was dissolved in 100 ml ethanol and added slowly to the olive oil-water mix. The final emulsion volume of 1000 mL was achieved by adding required amount of distilled water. The final product was stored in a 1 l dark polyethylene bottle. The final product had 50 mg of EOs per ml of

the emulsion medium. An EO-free emulsion was made and used for the control treatment.

The EOs emulsions were evaluated by dynamic light scattering technique to measure droplet size (nanoPartica SZ-100V2 Series, HORIBA Scientific Instrument, Kyoto, Japan) and by atomic force microscopy (CoreAFM Nanosurf, Liestal, Switzerland) to get a microscopic picture of the emulsion droplet sizes along with their distribution (Fig. 1). In addition, the surface charge density (zeta potential) assessment of the prepared emulsion droplets was performed using an electrophoretic light scattering technique (nanoPartica SZ-100V2 Series, HORIBA Scientific Instrument, Kyoto, Japan). Before the measurements, three samples were diluted to 5:100, 2:100 and 1:100 with distilled water according to the equipment optimal detection range and to avoid multiple scattering effects (Loeffler et al., 2014). All the measurements were repeated three times for each sample after 3 weeks of storage at room temperature to evaluate emulsion stability (Adjonu et al., 2014; Silva et al., 2015; Campelo et al., 2017).

2.2. Experimental design and animal management

Iranian Council of Animal Care guidelines (1995) were followed for animal handling and procedures. Forty male Holstein calves (7-day old; initial body weight of 40.4 ± 3.0 kg)

from Moghan Agro-Industrial and Animal Husbandry dairy herd (Parsabad, Ardabil, Iran) were randomly assigned to a completely randomized design with 4 treatments (n = 10): control (no EO), 100, 200, or 300 mg of a blend of EOs/d per calf (or 2, 4 or 6 ml of the emulsion per head and per day). Control calves received 4 ml of the emulsion medium with no EOs. For all the groups, a predetermined amount of the emulsion was incorporated into the morning milk meal by mixing before feeding from 7 days post-calving to weaning on d 42.

The calves were fed 4 kg of colostrum via a nipple pail after birth for 3 days and thereafter, they were fed 4 kg of whole milk daily in two meals at 8:00 and 18:00 for 2 weeks, 6 kg of whole milk daily for the third and fourth weeks, 4 kg of whole milk daily for the fifth week in two equal meals and 2 kg of whole milk daily only for morning meal for the sixth week before weaning (42 days). Buckets were used to deliver milk to the calves and the animals had free access to a starter diet and water from d 7. Chopped alfalfa was included in the diet from d 20 at the rate of 100 g/kg of the starter diet. Calves were housed in individual pens measuring 1 × 2.5 m/pen. The pens were cleaned and bedded daily with new dry straw.

2.3. Data collection, sampling procedure and chemical analysis

Calves were weighed biweekly on d 14, 28 and 42 of the experiment prior to morning meal. Feed intake was recorded daily by subtracting the orts from feed offered the previous day. Table 1 shows the ingredients and chemical composition of the starter diet, alfalfa hay, and milk. The starter diet and aflfalfa hay were analyzed for dry matter (method number 930.15), crude protein (Kjeldahl N × 6.25, method number 984.13), ether extract (method number 920.39), ash (method number 924.05; AOAC, 1990), neutral detergent fiber (NDF) and acid detergent fiber (ADF, Van Soest et al., 1991). The NDF was analyzed without a heat stable α-amylase but

with sodium sulphite. Both NDF and ADF were expressed inclusive of residual ash. Calcium and phosphorous content of starter diet and alfalfa hay were measured by atomic absorbtion spectroscopy (Atomic absorption spectrophotometer, AA-670, Shimadzu, Tokyo, Japan). Milk samples were analyzed by Delta dairy analyzer (CombiScope FTIR 600/300 Hp - Dairy Analyser, Delta Instruments, Drachten, Netherland) for dry matter, protein and fat content. Blood sampling was done via the jugular vein on d 15 and 30 of the experiment at approximately 3−4 h after morning feeding. Blood samples were collected in three separate tubes (two with sodium

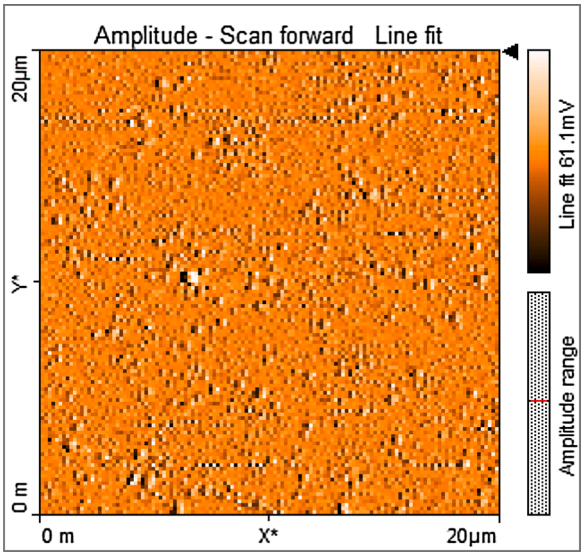

Fig. 1. Atomic force microscopy image of the prepared emulsion sample (after 2:100 dilution, v/v).

Table 1
Ingredients and chemical composition of the starter feed, alfalfa hay and milk (dry matter basis).

Item	Starter	Alfalfa hay	Hay
Ingredients, g/kg			
Corn, ground	400	–	–
Barley, ground	140	–	–
Wheat bran	50	–	–
Soybean meal	380	–	–
Salt	5	–	–
Calcium carbonate	10	–	–
Mineral and Vitamin premix[a]	10	–	–
Dicalcium phosphate	5	–	–
Chemical composition, g/kg			
Dry matter	902	897	124
Crude protein	190	148	33.1
Ether extract	28.5	23.4	36.8
Neutral detergent fiber	156	552	–
Acid detergent fiber	78	378	–
Calcium	6	14	–
Phosphorus	5.5	3	–

[a] Vitamin Premix provided per kg of diet: vit A, 200000 IU; vit D, 300000 IU; vit E, 10000 IU; vit K, 2 mg; Butylated hydroxytoluene 1000 mg/kg. Mineral premix provided per kg of diet: Cu, 3300 mg/kg; Fe, 100 mg; Zn, 16500 mg/kg; Mn, 9000 mg; I, 120 mg/kg; Co, 90 mg/kg; Se, 90 mg/kg.

heparin for plasma and whole blood samples, and one without heparin for serum, Novin Azma Pazhoohan, Ardabil, Iran). Blood samples were centrifuged at 3500 × *g* for 15 min at 4 °C and plasma and serum samples were frozen at −20 °C until used for analysis. Samples were thawed at room temperature and analyzed for glucose, cholesterol, total protein, albumin, globulin, blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) colorimetrically by using commercial kits (Pars Azmoon Co., Tehran, Iran). Beta-hydroxy butyric acid (BHBA) concentration in serum samples was measured by Ranbut assay (Ranbut assay, Randox Laboratories, Crumlin, UK). Glutathion peroxidase (GPx) and superoxide dismutase (SOD) activities were determined in whole blood (Ransel and Ransod kits from Randox Laboratories, Crumlin, UK). Total antioxidant status (TAS) was measured in serum samples by a commercially available kit according to the manufacturer's protocol (Total Antioxidant Status, Randox Laboratories, Crumlin, UK). Serum malondialdehyde (MDA) concentration was determined based on the method described by Moore and Robert (1998).

On d 30, fecal samples were collected from the rectum by inducing calves to defecate by inserting a moistened finger into the rectum and massaging until the external sphincter relaxes. Fecal samples were collected from all the calves for three consecutive days and analyzed in a microbiology laboratory (Zist Fanavaran Atye Tabriz, Tabriz) to estimate fecal *E. coli* and *Lacobacillus* spp. counts by violet red bile (VRB) agar (Manafi, 2003) and MRS (De Man, Rogosa and Sharpe) agar (Schillinger and Holzapfel, 2003) culture media, respectively.

2.4. Statistical analysis

Average daily gain and feed intake data were analyzed as a completely randomized design using repeated measurements analysis (where biweekly weighing of calves was used as a repeated measure) by the Mixed procedure of SAS (SAS Institute, 2003). The model included the effects of treatment, time and their interactions as fixed effects, and individual calves as a random effect. The UN covariance structure was used for repeated measures analysis based on its smaller Schwarz Bayesian criterion (Littell et al., 1998). The interactions were not significant so were removed from the model. Significant effect of time was observed for all performance data ($P < 0.01$). Linear and quadratic contrasts were done by using the orthogonal polynomial CONTRAST statement of SAS. Blood samples and fecal count data were analyzed as a completely randomized design using the GLM procedure of SAS with the effect of different levels of the emulsified EOs as treatment effect. Fecal count data were used after transformation to log 10. Initial body weight of calves was

Table 2
Droplet size and surface charge of the diluted samples.

Sample[a]	Droplet size (nm)[b]	Droplet size (nm)[c]	Z-average (nm)	Zeta potential (mV)	Electrophoretic Mobility (cm^2/Vs) × 10^{-4}
1	62–392	–	2785	−58.4	−4.52
2	121–768	100–300	1983	−73.9	−5.72
3	261–4628	100–300	942.8	−82.5	−6.39

[a] Sample 1) dilution 1:100, sample 2) dilution 2:100 and sample 3) dilution 5:100.
[b] Measured by dynamic light scattering technique.
[c] Measured by atomic force microscopy.

considered as a covariate for all analyses. $P \leq 0.05$ was considered as significant and $P < 0.10$ was considered to have a tendency to be significant.

3. Results

3.1. Emulsion characteristics of EOs

Measurements of emulsion characteristics taken after 3 weeks showed three types of particles with different diameters based on their dilution rate (Table 2). Numerically, there was an increase in particle size distribution with increasing emulsion concentration. To evaluate emulsion stability in terms of electrostatic contribution and dilution impact, zeta-potential of the samples were evaluated. Results of the samples showed that zeta-potential values ranged from −58 to −82 mV. To evaluate the EOs emulsion droplet size, spin-coated thin films of the samples were exposed to atomic force microscopy imaging as shown in Fig. 1. Apart from the 1:100 diluted sample which had no clear image, uniform distribution of spherical partials with different dimensions in the range of 100−300 nm, were captured for the other two emulsions. This was comparable with the dynamic light scattering results. The nature of the particle constituents of the emulsion did not change with concentration.

3.2. Calves growth performance

Supplementation of the emulsified EOs did not influence final body weight of the calves (Table 3). Calves daily gain from d 1−14 was not affected by the emulsified EOs. However, the emulsified EOs linearly ($P = 0.01$) increased average daily gain of calves on d 14−28 and d 28−42. Overall average daily gain was not affected by increasing doses of the emulsified blend of EOs. Daily feed intake and feed conversion ratio were not affected by feeding different levels of the emulsified blend of EOs.

3.3. Blood metabolites

Glucose, albumin, and cholesterol concentrations were not affected by the emulsified EOs (Table 4). However, triglyceride concentration linearly increased from d 15 ($P = 0.01$) and d 30 ($P = 0.02$) with increasing doses of the EOs. The concentration of blood beta-hydroxy butyric acid (BHBA) was not affected by emulsified EOs inclusion. On d 15, blood urea nitrogen (BUN) concentration decreased linearly ($P = 0.01$) with increasing doses of the emulsified EOs. On d 30, there was a tendency for a quadratic response ($P = 0.08$) on BUN concentration with the lowest BUN concentration noted in calves that received 100 mg EOs. Blood level of gamma glutamyl transferase (GGT) was affected by the experimental treatments with a linear decrease ($P = 0.01$) noted on d 15 and 30. Blood concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were not affected by EO supplementation on d 15. However, after 30 days, blood ALT and AST concentrations decreased linearly ($P = 0.01$) and the lowest ALT and AST concentrations were recorded for calves that received 300 mg of emulsified EOs.

Table 3
Effects of different levels of emulsified essential oils[a] on calves growth performance.

Item	CON	100E	200E	300E	SEM[c]	*P*-value	
						Linear	Quadratic
Initial body weight, kg	39.9	39.9	41.2	40.6	1.32	0.57	0.82
Final body weight, kg	59.1	60.4	60.7	62.3	1.50	0.15	0.93
ADG[b], g/d							
d 1−14	343	354	311	350	24.58	0.84	0.57
d 14−28	394	423	409	477	21.99	0.02	0.36
d 28−42	643	698	696	734	19.41	0.01	0.63
Overal	446	476	445	504	9.36	0.25	0.79
Feed intake, g/day							
d 1−14	117	109	111	113	7.58	0.72	0.52
d 14−28	299	292	314	317	15.95	0.29	0.70
d 28−42	702	743	730	743	27.36	0.38	0.58
Overal	373	381	385	391	48.98	0.79	0.98
FCR[b]							
d 1−14	0.36	0.32	0.37	0.34	0.033	0.87	0.84
d 14−28	0.77	0.70	0.76	0.69	0.042	0.33	0.97
d 28−42	1.11	1.07	1.05	1.02	0.047	0.16	0.99
Overal	0.75	0.70	0.73	0.68	0.058	0.50	0.97

[a] CON = control diet with no additive; 100E = control diet with 100 mg/d of emulsified EOs in milk, 200E = control diet with 200 mg/d of emulsified EOs in milk; 300E = control diet with 300 mg/d of emulsified EOs in milk.
[b] ADG = average daily gain; FCR = feed conversion ratio calculated as feed/gain without considering the consumed milk.
[c] SEM = standard error of the mean.

Table 4
Effects of different levels of emulsified essential oils[a] on blood metabolites of calves.

Item	CON	100E	200E	300E	SEM[c]	*P*-value	
						Linear	Quadratic
Glucose, mg/dl							
d 15	104.2	108.4	109.4	105.3	5.67	0.87	0.47
d 30	82.5	86.2	84.4	82.3	2.71	0.84	0.29
Total protein, g/dl							
d 15	6.2	5.9	6.5	6.1	0.13	0.73	0.69
d 30	6.2	6.1	6.3	6.1	0.12	0.93	0.97
Albumin, g/dl							
d 15	4.3	4.0	4.2	4.1	0.11	0.56	0.49
d 30	4.2	4.1	4.2	4.1	0.12	0.56	0.49
Triglyceride, mg/dl							
d 15	9.6	11.1	12.1	14.6	1.26	0.01	0.69
d 30	13.3	13.2	15.3	15.7	0.92	0.03	0.79
Cholestrol, mg/dl							
d 15	118.1	114.3	122.1	124.9	9.73	0.52	0.74
d 30	96.2	104.3	100.8	95.4	5.51	0.78	0.20
BHBA[b], mg/dl							
d 15	0.09	0.07	0.09	0.08	0.01	0.77	0.85
d 30	1.15	1.12	1.16	1.12	0.03	0.69	0.68
BUN[b], mg/dl							
d 15	21.2	17.5	17.3	16.7	0.89	0.01	0.09
d 30	29.1	25.6	28.0	29.3	1.35	0.62	0.08
ALT[b], U/ml							
d 15	9.6	9.1	9.0	9.4	0.85	0.86	0.60
d 30	13.6	13.3	12.4	11.1	0.58	0.01	0.41
AST[b], U/ml							
d 15	40.3	39.2	40.7	38.3	2.72	0.71	0.81
d 30	56.3	47.2	51.6	46.3	2.76	0.04	0.49
GGT[b], U/ml							
d 15	34.5	34.9	30.3	27.5	1.47	0.01	0.28
d 30	40.5	34.5	35.3	27.7	1.83	0.01	0.66

[a] CON = control diet with no additive; 100E = control diet with 100 mg/d of emulsified EOs in milk, 200E = control diet with 200 mg/d of emulsified EOs in milk; 300E = control diet with 300 mg/d of emulsified EOs in milk.
[b] BHBA = beta-hydroxy butyric acid, BUN = Blood urea nitrogen, AST = Aspartate aminotransferase, ALT = Alanine aminotransferase, GGT = Gamma glutamyl transferase.
[c] SEM = standard error of the mean.

3.4. Blood oxidative markers

Serum concentrations of GPx and SOD were not affected by feeding the blend of EOs (Table 5). However, blood SOD activity linearly ($P = 0.05$) increased with increasing doses of EOs on d 15 of the experiment. Malon di-aldehyde concentration decreased linearly ($P = 0.01$) with increasing doses of the emulsified blend of EOs on d 15 and 30. A linear ($P = 0.01$) increase in total antioxidant status (TAS) on d 15 of the experiment and a tendency ($P = 0.07$) for a linear increase on d 30 were noted in calves that received the blend of EOs. Calves that received 300 mg of emulsified EOs (300E) had the highest TAS in their blood samples.

3.5. Count of fecal E. coli and Lactobacillus

Feeding different doses of the emulsified EOs decreased *E. coli* shedding in a linear manner ($P = 0.01$) and calves that received 300 mg of EOs in emulsified form shed the lowest *E. coli* count in fecal samples (Table 6). Increasing amounts of the blend of EOs also resulted in a linear reduction ($P = 0.03$) of *Lactobacillus* counts in the feces.

Table 5
Effects of different levels of emulsified essential oils[a] on some blood oxidative markers.

Item[b]	CON	100E	200E	300E	SEM[c]	*P*-value	
						Linear	Quadratic
GPx, U/g Hb							
d 15	57.9	62.4	60.1	61.9	2.75	0.43	0.62
d 30	60.8	62.4	66.1	63.8	2.30	0.23	0.43
SOD, U/g Hb							
d 15	1642	1738	1788	1760	45.36	0.05	0.18
d 30	1722	1808	1748	1838	39.89	0.12	0.96
MDA, mmol/l							
d 15	1.97	1.73	1.57	1.56	0.116	0.01	0.33
d 30	2.17	1.73	1.63	1.65	0.120	0.01	0.06
TAS, mmol/l							
d 15	0.10	0.14	0.13	0.15	0.065	0.01	0.11
d 30	0.31	0.35	0.34	0.48	0.062	0.08	0.34

[a] CON = control diet with no additive; 100E = control diet with 100 mg/d of emulsified EOs in milk, 200E = control diet with 200 mg/d of emulsified EOs in milk; 300E = control diet with 300 mg/d of emulsified EOs in milk.
[b] GPx = Glutathion peroxidase, SOD = Super oxid dismutase, MDA = Malondialdehyde, TAS = Total antioxidant status, Hb = Hemoglobin.
[c] SEM = standard error of the mean.

Table 6
Effects of different levels of emulsified essential oils[a] on *E. coli* and *Lactobacillus* counts of feces.

Item,	CON	100E	200E	300E	SEM[b]	*P*-value	
						Linear	Quadratic
Feces microorganisms, $\log_{10}$ CFU/g dry matter							
E. coli count	5.07	5.02	4.72	4.47	0.126	0.01	0.43
Lactobacillus count	6.63	6.55	6.37	6.13	0.169	0.04	0.65

[a] CON = control diet with no additive; 100E = control diet with 100 mg/d of emulsified EOs in milk, 200E = control diet with 200 mg/d of emulsified EOs in milk; 300E = control diet with 300 mg/d of emulsified EOs in milk.
[b] SEM = standard error of the mean.

4. Discussion

Emulsion based delivery systems are extensively used in food and pharmaceutical industries to increase digestion, absorption, and to controlled-release and targeted delivery of bioactive components (Roohinejad et al., 2018). Inability to fully incorporate bioactive ingredients into foods or feeds will result in poor chemical stability, low bioavailability, high volatilization and low water solubility (Roohinejad et al., 2018) but this can be overcome by emulsification. Essential oils from medicinal plants are a group of phyto-chemicals with numerous health benefits; however, their application in food and drug industry is faced with some challenges due to their hydrophobic nature, low water solubility, high volatilization, low stability and low absorption in digestive tract.

Determination of the droplet size of the EOs emulsion at three dilution rates by dynamic light scattering technique showed two types of particle diameters which ranged from 62–392 nm at dilution rate of 1:100 and 261–4628 nm when dilution rate decreased to 5:100. The increase in average particle size distribution with increasing emulsion concentration or decreasing dilution rate was probably due to aggregation process of the droplets. Aggregation likely occurred with increasing sample concentration which was accompanied with broadening dynamic light scattering peaks (Bae and Lee, 2008; Sukhotu et al., 2016). Measurement of the diluted samples by atomic force microscopy imaging technique showed more consistent droplet sizes (100−300 nm). Conventional oil-in-water emulsions consist of oil droplets dispersed in an aqueous phase with mean diameter of the droplets between 100 nm to 100 μm. As shown in Table 2, the microdroplets possess a negative surface charge greater than 30 mV. It is known that emulsion systems with zeta-potential value of greater than ±60 mV are considered to possess excellent stability and the ones with zeta-potential greater than ±30 mV are considered to have good stability (Herculano et al., 2015). Based on the above classification, all the emulsions in the current study had excellent stability.

Increased average daily gain noted in the present study is consistent with previous studies (Hill et al., 2007; Jeshari et al., 2016). However, positive effect on ADG was recorded for d 14−28 and 28−42 and not for d 1−14. This may indicate that the EOs require some time to exert their effects on performance. Jeshari et al. (2016) reported greater average daily gain in calves fed a starter diet supplemented with 300 mg/kg of a blend of EOs from *R. officinalis, Z. miltiflora, and M. pulegium* and the highest daily gain was recorded after 5 weeks of feeding the EOs. Similarly, Salazar et al. (2019) reported that calves fed EOs or a probiotic had similar daily gain for the whole period (before weaning) compared to control calves. Calves fed the probiotic treatment recorded significant daily

gain for d 28−42 and calves that received EOs had better performance after weaning. In addition, Hill et al. (2007) observed that calves fed a commercial herbal blend had better growth performance on d 0–35 of the experiment but not for overall period (d 0–56). The main route that EOs may affect growth performance of suckling calves will be by their antimicrobial, antioxidant or anti-inflammatory effects (Oh et al., 2017). Therefore, these variations on growth performance may be dependent on the time that EOs require to affect growth performance or health challenges that may occur during the different stages of production. Elcoso et al. (2019) reported that cows fed a commercial blend of EOs produced more energy corrected milk after 4 weeks of exposure to the treatments.

In contrast, other studies did not observe any significant effect on the growth performance of suckling calves supplemented with plant derived EOs (Santos et al., 2015; Pempek et al., 2018; Salazar et al., 2019). Differences in these results were likely due to differences in the origin of the EOs, doses, route of feeding, and calf's health status. The positive effects of EOs from medicinal plants on livestock animal performance are mostly attributed to their antimicrobial, antioxidant, anti-inflammatory and immunomodulation properties. The main area of interest in the application of EOs in ruminant nutrition is on their effects to manipulate rumen fermentation (Calsamiglia et al., 2007). However, in non-ruminants, EOs had some positive health effects on the host animal by their effects on intestinal microbiota, immune system, general metabolism and blood antioxidant capacity. To exhibit similar effects in ruminants, it was proposed that EOs have to bypass ruminal microbial degradation (Oh et al., 2017). The ruminal bypass rates of capsaicin and di-hydrocapsaicin from *Capcicum oleoresin* were between 15 and 33 % depending on the dose (Oh et al., 2016). Use of an emulsion-based system to deliver EOs by milk, not only bypassed rumen degradation but also enhanced absorption of the EOs. Incorporation of lipophilic bioactive compounds such as plant derived EOs in emulsions is advantageous with resultant effects of enhanced palatability, absorption and bioactivity (McClements et al., 2007). The observed positive effects on average daily gain (ADG) could be due to a higher absorption rate or efficacy of the blend of EOs. The higher absorption rate will provide an opportunity to get favorable results with lower doses and lower costs. So, feeding 100 mg EOs in emulsified form resulted in similar growth rate compared to 200 mg EOs on d 28−42.

Plant derived EOs have physiological effects on hormone regulation, liver function, whole body metabolism and blood metabolites (Jeshari et al., 2016; Oh et al., 2017). Increased blood triglycerides, observed in the present study, may have been caused by the effects of the EOs on insulin secretion and sensitivity. Jeshari et al. (2016) observed elevated levels of blood insulin in suckling calves supplemented with EOs or distillation residues of three medicinal plants (*R. officinalis, Z. multiflora* Bioss. and *M. pulegium*). Increased blood insulin or increased tissue sensitivity to insulin by feeding EOs of medicinal plants or their other derivates has been reported previously by Oh et al. (2017).

Blood concentrations of liver enzymes such as AST, ALT, and GGT are indicators of liver function and its tissue integrity. Elevated levels of these enzymes in the blood indicate damage to liver cells by trauma, inflamation, or cell wall lipid oxidation. Results showed that feeding the emulsified EOs obtained from a blend of four medicinal plants lowered blood GGT level after 15 days and ALT and AST levels after 30 days. Medicinal plant derived EOs can serve as exogenous antioxidant to scavenge reactive oxygen species and prevent lipid oxidation (Amorati et al., 2013). This hypothesis is consistent with lower MDA concentration in the blood of calves that recieved the emulsified EOs compared to control calves. The MDA was produced from peroxidation of polyunsaturated fatty acids and used as an oxidative stress marker. Decreased MDA level by feeding EOs of *T. vulgaris* (Placha et al., 2014), *S. officinalis* (Placha et al., 2015) and turmeric and rosemary (Gharejanloo et al., 2017) have been reported earlier.

Endogenous antioxidants such as SOD and GPx are first lines of defense against reactive oxygen species and increased levels of these antioxidants indicate lower oxidative stress or higher exogenous antioxidant consumption (Oh et al., 2017). Antioxidant effects of EOs of *T. kotschyanus* (Morteza-Semnani et al., 2006), *L. angustifolia* (Cavanagh and Wilkinson, 2005), *S. officinalis* (Bozin et al., 2007), and *C. spinosa* (Tlili et al., 2010) have been reported earlier. Higher antioxidant consumption from the emulsified blend of EOs and higher SOD and GPx levels in the blood may explain the higher total antioxidant status (TAS) of the calves that received the emulsified blend of EOs. In addition, higher TAS may elucidate the lower levels of liver enzymes (ALT, AST and GGT) in the blood of calves supplemented with the emulsified blend of EOs because higher antioxidant capacity results in lower oxidative stress and therefore lower damage to tissues such as the liver (Oh et al., 2017). Moreover, significant linear effect of the different doses of the EOs showed that higher doses resulted in higher antioxidant status so, calves that received 300 mg EOs had the highest blood TAS and the lowest blood MDA, AST and GGT.

Antimicrobial effect is the most important characteristic of medicinal plant derived EOs that attracted the attention of researchers all over the world. Based on their potential, EOs have been proposed as a natural alternative to antibiotics use as growth promoters (Salazar et al., 2019). The emulsified blend of EOs derived from four medicinal plants was effective in lowering *E. coli* counts in fecal samples. The antimicrobial effects of the medicinal plants used in the present study against *E. coli* bacteria have been established by earlier studies (Yap et al., 2014; Asheghian Amiri et al., 2015). In addition, Moghimi et al. (2015) reported that nano-emulsion of *T. daenensis* EO had superior antibacterial activity against *E. coli* bacteria. They proposed that the mode of action of emulsified EOs is the disruption of bacteria cell wall or possibly altering the bacteria cell wall integrity or by interfering with active transport of proteins embedded in the bacteria cell wall. In the present study, results of the antibacterial effects of the emulsified EOs on *E. coli* are consistent with the previous studies mentioned above.

5. Conclusion

The present study was an attempt to apply emulsion technology to deliver EOs and enhance their effectiveness. The blend of medicinal plants used as emulsion was effective in improving blood antioxidant and liver integrity markers and lowering the *E. coli* shedding. Overall, emulsion-based delivery system is an efficient and convenient way to administer lipophilic compounds such as EOs to suckling calves. Findings of present study suggest that 300 mg/d/calf of the blended EOs can be used in emulsified form via milk

with positive effects on calves health.

Declaration of Competing Interest

None.

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