Effect of nanomicelles of *Thymus vulgaris*, *Carum copticum*, *Mentha longifolia*, and *Lavandula angustifolia* essential oils on the performance and health status of suckling calves

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ABSTRACT

This study investigated the effects of nanomicelles of four essential oils on functional and antioxidant parameters of 48 male Holstein newborn calves. The calves were assigned to six groups and monitored over a 46-day period. The groups included one control group and five treatment groups, each receiving 400 mg of a specific nanomicellized essential oil (NEO) – *Thymus vulgaris* (TNEO), *Carum copticum* (CNEO), *Mentha longifolia* (MNEO), *Lavandula angustifolia* (LNEO) – or a blend of all four (BNEO), administered daily via 8 ml of milk emulsion. No significant differences in average daily weight gain (ADG) were observed among the groups during days 0-14 and 14-32. However, during days 32-46, calves supplemented with TNEO and LNEO showed significantly higher ADG (P=0.004). Calves receiving LNEO exhibited significantly higher blood concentrations of total protein (P=0.022) and albumin (P=0.046) compared to both the control and other treatment groups. Alanine aminotransferase (ALT) levels were lower in the TNEO, LNEO, MNEO, and BNEO groups compared to the control (P=0.012). Blood glutathione peroxidase (GPx) (P=0.009) and superoxide dismutase (SOD) (P=0.001) activities were elevated in the TNEO and BNEO groups. Malondialdehyde (MDA) concentrations were reduced in all NEO-supplemented groups, with the lowest levels observed in the TNEO group (P=0.009). Although the control group exhibited the lowest total antioxidant capacity (TAC), no significant differences were detected among the NEO-treated groups. Overall, these findings suggest that nanomicellized essential oils of *Lavandula angustifolia* and *Thymus vulgaris* confer the most notable benefits to suckling calves, enhancing weight gain, feed consumption, blood parameters, and oxidative stress markers.

Introduction

Although antibiotics are effective in enhancing animal performance and health, their prolonged use raises concern due to the potential development of antibiotic-resistant microorganisms, which can lead to economic losses and treatment challenges. These resistant strains can act as reservoirs for resistance genes, posing a serious risk to public health (28). As a result, there is increasing interest in identifying alternative strategies that provide similar benefits to antibiotics but with fewer side effects and a lower risk of resistance development (39).

Essential oils (EOs) have emerged as promising alternatives due to their well-documented antioxidant, antimicrobial, and anti-inflammatory properties (7). *Thymus vulgaris*, from the Lamiaceae family, is a widely recognized aromatic herb known for its medicinal value, with its EO containing active compounds such as thymol, p-cymene, carvacrol, and γ-terpinene (24). *Lavandula angustifolia*, a perennial evergreen species, produces EO rich in linalool, linalool acetate, lavandolol, and γ-terpineol, exhibiting various biological and therapeutic activities (34). *Carum copticum*, belonging to the

Apiaceae family and the Trachyspermum genus (13), is another medicinal plant whose EO contains thymol, carvacrol, γ-terpinene, and p-cymene as major constituents (14). Similarly, *Mentha longifolia*, a perennial herb from the Lamiaceae family, is valued for its pharmacological properties, with its EO predominantly comprising carovene, limonene, iso-dihydro-carovene, and caryophyllene (9,11).

Despite their beneficial properties, EOs present several challenges, including volatility, hydrophobicity, and susceptibility to degradation by light, oxygen, and heat (21). To overcome these limitations, nanoemulsion-based encapsulation has been developed as a modern strategy to enhance the stability, bioavailability, and solubility of EOs (4, 16). Therefore, the present study aimed to determine the effects of nanomicelles containing essential oils of *Thymus vulgaris*, *Carum copticum*, *Mentha longifolia*, and *Lavandula angustifolia* on growth performance, blood parameters, and antioxidant status in suckling Holstein calves.

Materials and Methods

Preparation and Emulsification of Essential Oils: To create an oil-in-water emulsion, the lipid phase was composed of essential oils (Barij Essence Kashan, Iran), with lecithin serving as the emulsifier. Whey protein (Ehsan Confectionery, Ardabil, Iran) and gum Arabic (Sigma-Aldrich, CAS Number 9000-01-5) were incorporated as stabilizers. The EO blend was prepared by mixing equal proportions of EOs from four medicinal herbs. Nanomicellized essential oil (NEO) formulations were produced according to the method described by Asghari et al. (3). Briefly, the lipid phase was prepared by mixing 25 g of lecithin with 50 g of each essential oil. The mixture was stirred using a magnetic stirrer at 60°C for 10 minutes. In parallel, the aqueous phase was prepared by dissolving 2 g of gum Arabic and 10 g of whey protein in distilled water. This solution was homogenized at 3000 rpm for 15 minutes using a laboratory homogenizer. The lipid phase was then slowly added dropwise to the aqueous phase under gentle stirring. Distilled water was subsequently added to bring the total volume to 1000 ml. The final emulsions were stored in opaque plastic containers, with each milliliter containing 50 mg of essential oil. A control emulsion, identical in composition but lacking essential oils, was also prepared.

Characterization of Nanoemulsions: The droplet size distribution and surface charge density (zeta potential) of the nanoemulsions were analyzed using dynamic light scattering and electrophoretic light scattering techniques, respectively (nanoPartica SZ-100V2 Series, HORIBA Scientific Instrument, Kyoto, Japan). Prior to analysis, the emulsions were diluted 1:100 with distilled water. Measurements were performed in triplicate using three independently prepared samples.

Determination of Antioxidant Activity: The antioxidant activity of the nanoemulsions was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method (37). A 0.1 mmol DPPH solution was prepared in 99% methanol (Mayer, Mexico). For each test, 1 ml of the DPPH solution was added to 3 ml of 99% methanol in a test tube, followed by the addition of 50 µl of nanoemulsion. The mixture was incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a UV-visible spectrophotometer.

The percentage of free radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [(A_0-A_1)/A_0]\times 100$$

Where A_0 is the absorbance of the control (1 ml DPPH + 3.05 ml methanol) and A_1 is the absorbance of the sample. All measurements were performed in triplicate, and results are expressed as mean values.

Emulsion Characteristics: The physical stability of the emulsions was evaluated after 21 days under ambient conditions, and the results are listed in Table 1. Droplet size ranged from 151.9 to 213.9 nm, with a Z-average particle size between 153.8 and 398.4 nm. The zeta potential values varied from -27.0 to -48.6 mV. Results of the DPPH assay showed that nanoemulsions containing essential oils from *Carum copticum* and *Thymus vulgaris* exhibited greater free radical scavenging activity compared to those prepared with *Mentha longifolia* and *Lavandula angustifolia*.

Table 1. Emulsion characteristics and antioxidant activity of nanomicellized essential oils[a]

Traits	TNEO	CNEO	MNEO	LNEO	BNEO
Droplet size (nm)[b]	198.1	188.2	152.4	213.9	151.9
Z-average (nm)	174.8	197.2	398.4	184.7	153.8
Zeta potential (mV)	-27.0	-31.4	-48.6	-33.2	-48.5
Electrophoretic Mobility (cm2/Vs)×10^{-4}	-2.1	-2.4	-3.8	-2.6	-3.7
DPPH radical scavenging activity (%)	74.54	81.48	20.14	14.58	72.11

TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. [a]Samples: Dilution 1:100. [b]Measured by dynamic light scattering technique.

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Table 2. The chemical composition of the starter diet, alfalfa hay and milk (dry matter basis).

Item, g/kg	Starter[a]	Alfalfa hay	Milk
Dry matter	912	886	125
Crude protein	194	147	34.20
Ether extract	29.10	23.10	37.10
Neutral detergent fiber	150	561	-
Acid detergent fiber	77	375	-
Calcium	7.00	15.10	-
Phosphorus	5.60	2.90	-

[a] Starter diet contained 410 kg ground corn, 130 kg ground barley, 50 kg wheat bran, 380 kg soybean meal, 5 kg salt, 10 kg calcium carbonate, 5 kg dicalcium phosphate and 10 kg vitamin and mineral premix (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; Fe, 100 mg; Zn, 16500 mg; Mn, 9000 mg; I, 120 mg; Co, 90 mg; Se, 90 mg.

Experimental Design and Animal Management: A total of 48 male Holstein calves (average body weight: 43.02 ± 3.60 kg; average age: 12.08 ± 4.37 days) were randomly selected from the dairy herd of Moghan Agriculture and Animal Husbandry dairy herd (Parsabad, Ardabil, Iran). The calves were assigned to six experimental groups using a completely randomized design and monitored over a 46-day period. The groups included a control group and five treatment groups receiving 400 mg/day of nanomicellized essential oils: TNEO, CNEO, MNEO, LNEO, or a blend of all four nanomicellized essential oils (BNEO), administered via 8 ml of milk emulsion.

From day 7 of age until weaning on day 46, the specified emulsion dose was added to the calves' morning milk feeding. During the first three days after birth, each calf received 4 kg of colostrum per day via teat bucket. In the subsequent two weeks, they were fed 4 kg of milk daily, divided into two meals at 8:00 and 18:00. From weeks 3 to 5, milk allowance increased to 6 kg per day. In week 6, the amount was reduced to 4 kg per day (split into two feedings), and in week 7, calves received 2 kg of milk only in the morning, and weaning occurred on day 46. Milk was provided in buckets.

From day 7 onward, calves had ad libitum access to a calf starter (Table 1) and fresh water. From day 20, chopped alfalfa was introduced at 10% of the starter feed. Calves were individually housed in 1 × 2.5 m stalls. Bedding was inspected daily, and wet bedding was replaced with clean, dry straw.

Sampling and Analysis: Body weights of the calves were recorded on the first day of the experiment and then on days 14, 32, and 46, prior to the morning meal. The daily feed intake was calculated by subtracting feed refusals from the amount offered the previous day. The chemical composition of milk, starter feed, alfalfa hay, and milk is presented in Table 2. The starter diet and alfalfa hay were analyzed for dry matter (DM: AOAC (2) method 930.15), crude protein (CP: method 984.13, Kjeldahl N×6.25), ether extract (method 920.39), and ash (method 924.05). The fiber insoluble in neutral detergent (NDF) and fiber insoluble in acidic detergent (ADF) were measured according to Van Soest et al. (38). NDF analysis was conducted without the use of heat-stable alpha-amylase but in the presence of sodium sulfite. Calcium and phosphorus concentrations in the starter and hay were measured using atomic absorption spectrophotometry (AA-670, Shimadzu, Tokyo, Japan). Milk composition (DM, CP, and fat) was determined using a milk analyzer (CombiScope FTIR 600/300 Hp - Dairy Analyser, Delta Instruments, Drachten, Netherlands).

Fecal Scoring and Health Monitoring: Fecal consistency was scored daily using the 5-point scale described by Khan et al. (17): score 1: hard and tubular feces; score 2: soft to loose; score 3: loose to watery; score 4: watery with mucus and traces of blood; and score 5: watery with mucus and visible blood. Calf health was assessed daily following the criteria of Lowe et al. (22), including visual evaluation of appearance, nasal and eye secretions, eye depression, and rectal temperature.

Microbiological Analysis: In the third and sixth weeks of the experiment, feces were collected rectally from each calf to assess *Escherichia coli*. Fecal samples were cultured on eosin methylene blue agar for coliform enumeration. Plates were incubated inverted at 37°C for 48 hours. Colonies were counted using a digital colony counter, and results were expressed as $\log_{10}$ colony-forming units per gram of dry matter ($\log_{10}$CFU/g DM).

Blood Sampling and Biochemical Analysis: On day 20, approximately 3 ml of blood was collected from the jugular vein 4 hours post-feeding in the morning. Blood was drawn into tubes with and without sodium heparin (Pars Azmoon Co., Tehran, Iran). Blood samples were centrifuged at 3000 rpm for 15 minutes at 4 °C and were kept at -20°C and thawed at room temperature before analysis. Serum biochemical parameters, including glucose, cholesterol, triglycerides, total protein, albumin,

aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were measured using commercial kits (Pars Azmoon Co., Tehran, Iran). After measuring total hemoglobin, blood samples were centrifuged at 4000 rpm for 10 minutes. The buffy coat and plasma layers were gently separated, and the erythrocytes were washed three times with 0.9% saline solution. Hemolysates were then prepared and used for the analysis of GPx and SOD activities using Ransel and Ransod kits, respectively (Randox Laboratories, Crumlin, UK).

Hematological and Oxidative Status Analysis: Hematological parameters, including red blood cell (RBC) count, hemoglobin concentration, hematocrit (HCT), white blood cell (WBC) count, neutrophils, lymphocytes, monocytes, and eosinophils, were measured using an automatic cell counter (Sysmex XT-2000iV analyzer, Japan) following the manufacturer's protocol with bovine-specific settings.

Serum total antioxidant status (TAS) was assessed using a commercial kit (Randox Laboratories, Crumlin, UK). Serum MDA levels were determined according to the method described by Moore and Robert (26) as an indicator of lipid peroxidation.

Statistical Analysis: Data on average daily gain and feed intake were analyzed using a repeated-measures ANOVA in a completely randomized design. Calf body weight at biweekly intervals was considered a repeated measure using the MIXED procedure of SAS (35). The model included treatment, time, and their interaction as fixed effects, with individual calf as a random effect. Non-significant interactions were removed from the final model. Time had a significant effect on all performance parameters ($P < 0.01$).

Blood samples were analyzed using a completely randomized design with the General Linear Model (GLM) procedure in SAS, considering the different levels of emulsified essential oils as the treatment effect. Initial body weight was included as a covariate in all models. Differences were considered statistically significant at $P \leq 0.05$, and trends toward significance were noted when $P < 0.10$.

Results

As shown in Table 3, supplementation of milk with NEOs did not significantly influence the final body weight of calves. There were no significant differences in ADG among treatment groups during days 0–14 and 14–32. However, during the 32–46 day period, calves receiving TNEO and LNEO exhibited significantly higher ADG compared to the other groups ($P = 0.004$). Additionally, the total ADG over the experimental period was significantly higher in calves supplemented with LNEO, TNEO, and BNEO than in the control group ($P = 0.018$), with the highest value observed in the LNEO group.

Table 3. Effects of different nanomicellized essential oils on feed intake and growth performance of suckling calves.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
Initial weight (kg)	43.9	43.7	43.7	43.7	43.8	43.7	1.78	1.000
Final weight (kg)	65.9	70.7	69.2	67.3	71.8	69.9	2.03	0.374
ADG (g/d)								
0-14 days	337.2	390.5	433.3	378.6	402.9	394.0	0.03	0.394
14-32 days	512.5	587.5	565.0	532.5	638.0	615.8	0.04	0.243
32-46 days	^{b}612.5	^{a}808.3	^{b}677.8	^{b}643.1	^{a}805.0	^{b}698.6	0.03	0.004
Total	^{c}523.4	^{ab}640.9	^{abc}607.1	^{bc}563.5	^{a}668.1	^{ab}624.2	0.03	0.018
Average daily feed intake (g/d)								
0-14 days	146.6	144.3	169.0	154.3	167.5	176.9	0.02	0.780
14-32 days	377.9	379.5	392.7	362.9	416.6	435.5	0.04	0.816
32-46 days	^{ab}944.0	^{ab}927.9	^{b}813.8	^{ab}910.9	^{a}1039.4	^{ab}953.1	0.07	0.032
Total	489.5	483.9	458.5	476.0	541.2	521.8	0.03	0.595
FCR								
0-14 days	0.44	0.39	0.40	0.41	0.42	0.49	0.06	0.883
14-32 days	0.74	0.65	0.72	0.69	0.69	0.69	0.07	0.961
32-46 days	^{b}1.54	^{a}1.16	^{a}1.22	^{ab}1.43	^{ab}1.30	^{ab}1.34	0.08	0.038
Total	^{b}0.94	^{a}0.76	^{a}0.76	^{ab}0.84	^{ab}0.82	^{ab}0.83	0.04	0.083

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. ADG = average daily gain; FCR = feed conversion ratio calculated as feed/gain without considering the consumed milk. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

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Table 4. Effects of different nanomicellized essential oils on blood metabolites and enzymes.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
Glucose, mg/dl	116.00	113.17	119.17	111.83	126.83	116.17	9.24	0.992
Cholesterol, mg/dl	114.00	107.67	138.83	115.00	124.83	124.17	10.86	0.453
Triglyceride, mg/dl	35.33	29.33	28.17	34.67	35.50	31.17	3.37	0.154
Total protein, g/dl	6.25^{b}	6.47^{b}	6.47^{b}	6.30^{b}	7.10^{a}	6.67^{ab}	0.19	0.022
Albumin, g/dl	2.95^{b}	2.92^{b}	3.00^{ab}	2.90^{b}	3.22^{a}	2.88^{b}	0.08	0.046
AST, U/ml	53.83	50.50	50.00	58.50	39.50	52.83	6.54	0.722
ALT, U/ml	13.17^{b}	10.17^{a}	11.83^{ab}	10.33^{a}	9.83^{a}	10.50^{a}	0.65	0.012

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. AST = Aspartate aminotransferase, ALT = Alanine aminotransferase. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

Table 5. Effects of different nanomicellized essential oils on blood oxidative parameters and antioxidant status.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
GPx, U/g Hb*	65.00^{b}	79.10^{a}	69.85^{ab}	72.28^{ab}	73.92^{ab}	76.05^{a}	3.11	0.009
SOD, U/g Hb	1067.78^{b}	1339.25^{a}	1324.58^{a}	1292.40^{a}	1274.87^{a}	1305.13^{a}	55.29	0.001
MDA, mmol/l	2.50^{b}	0.97^{a}	1.75^{ab}	2.03^{b}	1.65^{ab}	1.65^{ab}	0.32	0.009
TAC, mmol/l	0.38^{b}	0.47^{a}	0.49^{a}	0.55^{a}	0.48^{a}	0.48^{a}	0.03	0.002

CON= control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. GPx= Glutathione peroxidase, SOD= Superoxide dismutase, MDA= Malondialdehyde, TAC= Total antioxidant capacity. *Hb= Hemoglobin. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

Feed intake did not differ significantly among groups during days 0–14 and 14–32. However, from days 32 to 46, feed intake was significantly increased in calves supplemented with NEOs, with the LNEO group showing the highest consumption ($P = 0.032$). Furthermore, supplementation with NEOs significantly improved the feed conversion ratio (FCR) during both the 32–46 day period and the overall trial period. Specifically, calves in the CNEO and TNEO groups had lower FCRs compared to control calves ($P = 0.038$).

As presented in Table 4, blood concentrations of glucose, cholesterol, and triglycerides were not significantly affected by NEO supplementation. However, calves receiving LNEO exhibited significantly higher total protein levels compared to those in the control, TNEO, CNEO, and MNEO groups ($P = 0.022$). Similarly, LNEO supplementation led to a significant increase in blood albumin levels ($P = 0.046$). While NEO supplementation did not significantly alter AST levels, ALT concentrations were significantly lower in calves receiving TNEO, LNEO, MNEO, and BNEO compared to the control group ($P = 0.012$).

Dietary supplementation with NEOs significantly enhanced the activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) enzymes (Table 5). GPx activity was significantly higher in the TNEO and BNEO groups compared to the control group ($P = 0.009$), and all NEO-supplemented groups showed significantly higher SOD activity than the control ($P = 0.001$). Moreover, NEO supplementation reduced serum MDA concentrations, with the lowest MDA level recorded in the TNEO group ($P = 0.009$). Total antioxidant capacity (TAC) of the blood was also significantly improved by NEO supplementation ($P = 0.002$); control calves had the lowest TAC values, while no significant differences were observed among the NEO-treated groups.

There were no significant differences among experimental groups in terms of red blood cell (RBC) count, white blood cell (WBC) count, or hemoglobin (Hgb) concentration (Table 6). Similarly, differential counts of immune cells—including neutrophils, lymphocytes, monocytes, eosinophils, and basophils—were not significantly affected by NEO supplementation.

Calves' general appearance, nasal secretions, rectal temperature, and fecal *Escherichia coli* counts were not significantly influenced by dietary NEOs. However, fecal consistency scores were significantly affected by NEO supplementation, with treated calves displaying higher fecal consistency scores compared to the control group ($P=0.032$).

Table 6. Effects of different nanomicellized essential oils on hematological parameters.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
RBC (10^6/ul)	6.96	7.03	7.58	7.17	7.59	7.58	0.63	0.953
Hgb (g/dl)	7.55	7.28	7.22	8.22	7.83	8.05	0.75	0.912
WBC (10^3/ul)	7.02	8.22	7.51	8.94	7.77	7.13	1.00	0.757
Neutrophil (%)	45.54	37.66	39.42	43.61	39.21	38.65	4.83	0.829
Lymphocyte (%)	52.29	60.22	58.41	54.66	58.72	59.16	4.86	0.845
Monocyte (%)	0.93	1.21	1.19	0.93	1.08	1.35	0.31	0.921
Eosinophil (%)	0.91	0.70	0.73	0.59	0.61	0.70	0.21	0.907
Basophils (%)	0.31	0.22	0.24	0.22	0.39	0.15	0.12	0.762

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. RBC = Red Blood Cell, Hgb= Hemoglobin, Hct= Hematocrit, WBC= White blood cell. SEM = standard error of the mean.

Table 7. Effects of different nanomicellized essential oils on health indicators of suckling calves.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
Appearance	0.00	0.07	0.03	0.10	0.03	0.07	0.042	0.258
Eye discharge	1.01	0.90	0.70	0.63	0.77	1.07	0.227	0.518
Nasal discharge	0.00	0.03	0.10	0.03	0.00	0.03	0.043	0.287
Rectal temperature (°C)	38.95	38.90	39.02	38.97	38.83	38.80	0.078	0.606
Fecal consistency	2.73^{a}	1.92^{ab}	1.76^{b}	1.73^{b}	1.54^{b}	1.94^{ab}	0.120	0.032
Feces microorganisms, log10 CFU/g dry matter								
Escherichia coli count	6.48	6.40	6.26	5.84	6.40	6.53	0.258	0.776

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

Discussion and Conclusion

Essential oils, as secondary metabolites derived from medicinal plants, have attracted considerable interest in animal nutrition due to their broad-spectrum biological activities, including antibacterial, antifungal, anticoccidial, and antioxidant effects (40). Nevertheless, their practical use in livestock production remains limited due to their lipophilic nature and low water solubility, particularly in liquid feed systems such as milk (5). Nanoemulsion technology offers a promising approach to overcome these limitations by enhancing EO solubility, stability, and bioavailability. It also protects active compounds from degradation and increases cellular uptake, thereby improving antimicrobial and antioxidant efficacy (10).

In this study, EO-based nanoemulsions were successfully formulated with droplet sizes ranging from 151.9 to 213.9 nm, which fall within the accepted nanometric scale (20–200 nm) (15). The zeta potential values of these emulsions ranged from –0.27 to –48.6 mV. Emulsions with absolute zeta potential values greater than 30 mV are typically considered physically stable due to electrostatic repulsion between particles (8). These physical properties suggest that the formulated NEOs were suitably stable for oral administration in milk.

Essential oils (EOs) derived from medicinal plants are complex mixtures, primarily composed of terpenoids along with smaller amounts of non-terpenoid compounds. These constituents are well recognized for their antibacterial, anti-inflammatory, and antioxidant activities. Although the antioxidant potential of EOs has been widely documented, their anti-inflammatory and antihypertensive effects are often attributed to these antioxidant mechanisms (6). However, conventional chemical assays that assess redox activity—such as the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method—may not accurately represent the true antioxidant capacity of EOs in biological systems. The DPPH assay relies on the neutralization of a stable free radical and is frequently used to evaluate the *in vitro* antioxidant potential of plant extracts, including EOs (37). In the present study, *Lavandula angustifolia* and *Mentha longifolia* EOs demonstrated relatively low DPPH scavenging activity. The relatively low antioxidant activity observed for *Lavandula angustifolia* and *Mentha longifolia* essential oils in the present study aligns with the findings of Chen et al. (6), who reported that the DPPH radical scavenging capacity of essential oils is largely influenced by their chemical composition. Specifically, essential oils rich in thymol and eugenol exhibit strong

antioxidant activity in DPPH assays, whereas those dominated by linalool and menthol—such as *L. angustifolia* and *M. longifolia*—demonstrate comparatively weaker radical scavenging potential.

The present study demonstrated improved ADG in calves supplemented with NEOs, consistent with previous findings (3, 33). In particular, supplementation with *Lavandula angustifolia* and *Mentha longifolia* NEOs resulted in significantly higher ADG compared to other treatment groups. These findings are in agreement with Pawar et al. (30), who reported increased weight gain in buffalo calves fed *Carum copticum* seed oil. Similarly, Asghari et al. (3) showed that emulsified blends of essential oils derived from *Thymus vulgaris*, *L. angustifolia*, *Salvia officinalis*, and *Capparis spinosa* improved growth performance in suckling dairy calves. Moreover, Pawar et al. (30) observed enhanced dry matter intake in calves receiving *C. copticum* EO, supporting the potential of EO supplementation in improving feed efficiency and weight gain.

In line with the performance data, NEO supplementation was associated with reductions in blood levels of hepatic enzymes, particularly ALT and, to a lesser extent, AST. Elevated levels of AST and ALT are widely recognized biomarkers of hepatic stress or damage (23, 31). While AST levels were not significantly affected by treatment, ALT concentrations were significantly lower in NEO-supplemented calves than in controls. Given that ALT activity is more specific to hepatocellular damage due to its higher abundance in liver cells compared to AST, it serves as a more sensitive indicator of liver health (25). The reduction in liver enzyme levels in NEO-fed calves is consistent with the hepatoprotective effects of essential oils, which are largely attributed to their antioxidant activity (32, 36). These findings are further supported by previous *in vivo* and *ex vivo* studies demonstrating the protective effects of *L. angustifolia* EO on hepatic cells in both rodent and human models (18, 36). The superior growth performance observed in the LNEO group may thus reflect improved liver function and reduced oxidative stress.

Although NEO supplementation did not significantly alter blood concentrations of glucose, cholesterol, or triglycerides, a notable increase in total protein and albumin levels was observed in the LNEO group. The liver is the primary site for the synthesis of albumin and other serum proteins, and elevated levels of these biomarkers are indicative of enhanced liver function and metabolic health. Therefore, the increased blood protein concentrations in calves receiving LNEO may further support the notion of improved hepatic integrity and overall physiological status in this group.

SOD and GPx are key enzymatic antioxidants that play a central role in cellular defense against oxidative stress by scavenging reactive oxygen species (ROS) and neutralizing free radicals (20). Oxidative stress arises when the equilibrium between pro-oxidant and antioxidant systems is disturbed, leading to the accumulation of oxidative damage markers such as lipid peroxides in tissues (19). In the present study, calves supplemented with NEOs exhibited elevated blood levels of SOD and GPx, suggesting an enhancement of systemic antioxidant capacity and a reduction in oxidative stress. Among the treatment groups, the highest SOD and GPx activities were observed in calves receiving nanoemulsions of TNEO and CNEO. These increases were consistent with lower MDA concentrations and higher TAC in blood, as corroborated by the DPPH radical scavenging assay. The findings reflect the potent antioxidant properties of *T. vulgaris* and *C. copticum* essential oils, particularly in their nanoemulsified forms, which enhance their bioavailability and efficacy. These observations align with previous results reported by Asghari et al. (3), who demonstrated that supplementation with emulsified essential oils in suckling calves reduced lipid peroxidation (as indicated by decreased MDA levels) and elevated systemic antioxidant capacity (increased TAC). In addition to their direct antioxidant effects as exogenous radical scavengers (1), essential oils have also been shown to modulate the expression of genes involved in xenobiotic metabolism and endogenous antioxidant defense systems (27), further contributing to their protective role against oxidative stress at the molecular level.

The gastrointestinal tract of neonatal animals is colonized by *Escherichia coli* (E. coli) shortly after birth through environmental exposure. These bacteria constitute a significant component of the commensal intestinal microbiota throughout the animal's life. Although many *E. coli* strains are of low pathogenicity, they can act as opportunistic pathogens, causing extraintestinal infections such as wound infections, pneumonia, meningitis, and septicemia under certain conditions (29). In the present study, the absence of significant changes in fecal *E. coli* counts following NEO supplementation was consistent with the lack of significant differences in clinical health parameters, including general appearance, nasal secretions, and rectal temperature. This suggests that while NEOs may not drastically alter gut microbial load, they contribute positively to health status through their antioxidative, antimicrobial, and anti-inflammatory properties (7). Furthermore, the enhanced ADG observed in NEO-supplemented calves may be attributed to several mechanisms, including stimulation of antioxidant and anti-inflammatory pathways (12), preservation of liver integrity, modulation of metabolism (29), and potentially increased fecal shedding of *E. coli* as reported in previous

studies (3). Collectively, these findings support the potential of essential oils, particularly in nanoemulsified form, as viable alternatives to conventional antibiotics in livestock production.

In conclusion, this study evaluated the effects of essential oils (EOs) from four medicinal plants formulated into a water-soluble nanomicellized form and administered via milk to suckling calves. Supplementation with nanoemulsified essential oils from *Lavandula angustifolia*, *Mentha longifolia*, and a four-plant blend significantly improved ADG, with the highest growth performance observed in the *L. angustifolia* NEO (LNEO) group. LNEO supplementation also led to increased serum levels of total protein and albumin, alongside a reduction in liver enzyme concentrations (ALT and AST), suggesting enhanced liver function. Additionally, all NEO treatments—most notably those containing *Thymus vulgaris* EO—enhanced antioxidant defense, as evidenced by elevated blood levels of GPx, SOD, and TAC, along with decreased MDA concentrations. These findings suggest that nanomicellized essential oils derived from *Lavandula angustifolia* and *Mentha longifolia* represent promising dietary supplements for suckling calves, exerting beneficial effects on daily weight gain, feed intake, blood metabolic profiles, and oxidative stress biomarkers.

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Ethical Statement

Protocols applied for this experiment were approved by the Animal Ethics Committee of the University of Mohaghegh Ardabili (Ardabil, Iran) (Approval Number: IR.UMA.REC.1402.099) and a cooperation contract was signed between the University of Mohaghegh Ardabili and Moghan Agro-Industrial and Animal Husbandry Company for the participation of the animals.

Conflict of Interest

The authors declare that when conducting their search, there were no business or financial relationships that may be interpreted as constituting a conflict of interest.

Author Contributions

MAA, HAB, JS designed the experiment, carried out the research and laboratory analysis. HAB, JS supervision the student, RS, MAA, HAB, JS did the data analysis, wrote the manuscript, and revised the manuscript. All authors reviewed and agreed on the final manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Amorati R, Foti MC, Valgimigli L** (2013): *Antioxidant activity of essential oils.* J Agric Food Chem, **61**, 10835–10847.
2. **AOAC** (2000): Method 973.18. Official methods of analysis. Association of Official Analytical Chemists, International, Gaithersburg, MD, USA.
3. **Asghari M, Abdi-Benemar H, Maheri-Sis N, et al** (2021): *Effects of emulsified essential oils blend on performance, blood metabolites, oxidative status and intestinal microflora of suckling calves.* Anim Feed Sci Technol, **277**, 114954.
4. **Baskara AP, Ariyadi B, Dono ND, et al** (2020): *The Potential use of essential oil nanoemulsion as a novel alternative to antibiotics in poultry production-A review*. Iran J Appl Anim Sci, **10,** 203-212.
5. **Chen H, Davidson PM, Zhong Q** (2014): *Impacts of sample preparation methods on solubility and antilisterial characteristics of essential oil components in milk*. Appl Environ Microbiol, **80**, 907-916.
6. **Chen X, Shang S, Yan F, et al** (2023): *Antioxidant activities of essential oils and their major components in scavenging free radicals, inhibiting lipid oxidation and reducing cellular oxidative stress.* Molecules, **28**, 4559.
7. **Costa DC, Costa HS, Gonçalves Albuquerque T, et al** (2015): *Advances in phenolic compounds analysis of aromatic plants and their potential applications.* Trends Food Sci Technol, **45**, 336-354
8. **Da Costa S, Basri M, Shamsudin N, et al** (2014): *Stability of positively charged nanoemulsion formulation containing steroidal drug for effective transdermal application.* J Chem, **2014**, 1-8.
9. **Dadkhah A, Fatemi F, Rasooli A, et al** (2018): *Assessing the effect of Mentha longifolia essential oils on COX-2 expression in animal model of sepsis induced by caecal ligation and puncture.* Pharm Biol, **56**, 495-504.
10. **Donsi F, Annuanziata M, Sessa M, et al** (2011): *Nano-encapsulation of essential oils to enhance their antimicrobial activity in foods.* Food Sci Technol, **44**, 1908–1914.
11. **Farzaei MH, Bahramsoltani R, Ghobadi A, et al** (2017): *Pharmacological activity of Mentha longifolia and its phytoconstituents.* J Tradit Chin Med, **37**, 710-20.
12. **Favaretto JA, Alba DF, Marchiori MS, et al** (2020): *Supplementation with a blend based on micro-encapsulated carvacrol, thymol, and cinnamaldehyde in lambs feed inhibits immune cells and improves growth performance.* Livest Sci, **240**, 104144.

13. **Fazeli-Nasab B, Fooladvand Z** (2016): *A review on Iranian Carum copticum (L.): composition and biological activities.* European J Med Plants, **12**, 1-8.
14. **Ghadimian S, Esmaeili F** (2016): *Chemical composition of the essential oils of Carum copticum.* J Essent Oil-Bear Plants, **19**, 1834-1836.
15. **Jaiswal M, Dudhe R, Sharma PK** (2015): *Nanoemulsion: an advanced mode of drug delivery system.* 3 Biotech, **5**, 123–127.
16. **Khalili B, Abdi-benemar H, Seifdavati J, et al** (2024): *Micellized conjugated linoleic acid as an immune modifier feed additive for suckling calves.* Ankara Univ Vet Fak Derg, **71,** 445-52.
17. **Khan MA, Lee HJ, Lee WS, et al** (2007): *Starch source evaluation in calf starter: I. feed consumption, body weight gain, structural growth, and blood metabolites in Holstein calves.* J Dairy Sci, **90**, 5259- 5268.
18. **Kozics K, Srancikova A, Sedlackova E, et al** (2017): *Antioxidant potential of essential oil from Lavandula angustifolia in in vitro and ex vivo cultured liver cells.* Neoplasma, **64**, 485-93.
19. **Kuyumcu F, Aycan A** (2018): *Evaluation of oxidative stress levels and antioxidant enzyme activities in burst fractures*. Med Sci Monit, **24**, 225-234.
20. **Li J, Lei J, He L, et al** (2019): *Evaluation and monitoring of superoxide dismutase (SOD) activity and its clinical significance in gastric cancer: a systematic review and meta-analysis.* Med Sci Moni, **25**, 2032-2042.
21. **Liew SN, Utra U, Alias AK, et al** (2020): *Physical, morphological and antibacterial properties of lime essential oil nanoemulsions prepared via spontaneous emulsification method*. LWT, **128**, 109388.
22. **Lowe GL, Sutherland MA, Waas JR, et al** (2019): *Physiological and behavioral responses as indicators for early disease detection in dairy calves.* J Dairy Sci, **102**, 5389-5402.
23. **Mahmudul Hasan KM, Tamanna N, Anwarul Haque M** (2018): *Biochemical and histopathological profiling of Wistar rat treated with Brassica napus as a supplementary feed.* Food Sci Hum Wellness, **7**, 77-82.
24. **Mandal S, DebMandal M** (2016): Essential Oils in Food Preservation, Flavor and Safety, 1st Ed, 825-834. Department of Nutrition and Dietetics, King's College London, London, UK.
25. **McGill MR** (2016): *The past and present of serum aminotransferases and the future of liver injury biomarkers.* EXCLI J, **15**, 817-828.
26. **Moore K, Roberts LJ** (1998): *Measurement of lipid peroxidation*. Free Radic Res, **2**, 659-671.
27. **Mueller K, Blum NM, Kluge H, et al** (2012): *Influence of broccoli extract and various essential oils on performance and expression of xenobiotic and antioxidant enzymes in broiler chickens.* Br J Nutr, **108**, 588-602.
28. **Nhung NT, Chansiripornchai N, Carrique-Mas JJ** (2017): *Antimicrobial Resistance in Bacterial Poultry Pathogens: A Review*. Front Vet Sci, **4**, 126.
29. **Oh J, Wall EH, Bravo DM, et al** (2017): *Host-mediated effects of phytonutrients in ruminants: a review*. J Dairy Sci, **100**, 5974–5983.
30. **Pawar MM, Kamra DN, Chaudhary LC, et al** (2019): *Nutrient's utilization, methane emission, immune function, blood metabolites and performance of buffalo calves fed Trachyspermum copticum seed oil*. Indian J Anim Sci, **89**, 63–67.
31. **Rezaei Sarteshnizi F, Abdi-Benemar H, Seifdavati J, et al** (2020): *Influence of spray-dried rumen fluid supplementation on performance, blood metabolites and cytokines in suckling Holstein calves.* Animal, **14**, 1849-56.
32. **Rostami R, Eslamifar Z, Nazemi S, et al** (2022): *The effect of thyme essential oil on liver ınjuries caused by renal ıschemia-reperfusion in Rats.* Biomed Res Int, **2022**, 2988334.
33. **Salazar LFL, Nero LA, Campos-Galvao MEM, et al** (2019): *Effect of selected feed additives to improve growth and health of dairy calves.* PLoS One, **14**, e0216066.
34. **Sandner G, Heckmann M, Weghuber J** (2020): *Immunomodulatory activities of selected essential oils.* Biomolecules, **10**, 1139.
35. **SAS Institute** (2003): SAS User's Guide: Statistics, Release 9.1. SAS Inst, Inc, Cary, NC.
36. **Selmi S, Jallouli M, Gharbi N, et al** (2015): *Hepatoprotective and renoprotective effects of lavender (Lavandula stoechas L.) essential oils against malathion-induced oxidative stress in young male mice.* J Med Food, **18**, 1103-11.
37. **Sundararajan B, Moola AK, Vivek K, et al** (2018): *Formulation of nanoemulsion from leaves essential oil of Ocimum basilicum L. and its antibacterial, antioxidant and larvicidal activities (Culex quinquefasciatus).* Microb Pathog, **125**, 475-485.
38. **Van Soest PJ, Robertson JB, Lewis BA** (1991): *Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition.* J Dairy Sci, **74**, 3583-3597.
39. **Vizioli J, Salzet M** (2003): *Antimicrobial peptides: new weapons to control parasitic infections?* Trends Parasitol, **19**, 32-40
40. **Volpato A, Crecencio RB, Tomasi T, et al** (2019): *Phytogenic as feed additive for suckling dairy calves' has a beneficial effect on animal health and performance.* An Acad Bras Ciênc, **91**, e20180747.