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The Effects of Oregano Essential Oil and Sodium Butyrate on the Growth Performance, Blood and Rumen Fermentation Parameters of Suckling Holstein Calves

Research Article

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ABSTRACT

This study examined the effects of oregano essential oil (OEO) and sodium butyrate (SB) dissolved in milk on Holstein suckling calves' performance, and blood and fermentation parameters. Twenty-four newborn male calves (3 to 5 days old) weighing an average of 36 ± 2 kg were randomly divided into four equal groups. Experimental treatments included: 1- basal diet (control), 2- basal diet + 5 g SB, 3- basal diet + 5 g OEO, and 4- basal diet + 5 g SB and 5 g OEO. Every two weeks, calves were weighed, and their skeletal growth indices were measured. In addition, the daily feed intake was determined. Blood samples were taken on days 7, 21, 42, and 56, and analyzed for determination of serum glucose, albumin, total protein, β-hydroxybutyrate (BHBA), total cholesterol, triglycerides, and urea concentration. On the 21st, 42nd, and 56th days of the experiment, rumen fluid was collected to determine pH, ammonia nitrogen (NH_3-N), and volatile fatty acids (VFAs) concentrations. The SB and SB + OEO treatments increased feed intake compared to the control (P=0.06). Neither weight gain (P=0.11) nor feed conversion ratio (FCR, P=0.45) were influenced by treatments. However, some skeletal growth characteristics, including body length, withers height, and hip height, were enhanced by SB and OEO (P=0.05). Except protein, which was reduced by all treatments (P=0.002), there were no significant differences in blood parameters among treatments. Although treatments with SB and OEO increased the concentrations of acetic acid, propionic acid, butyric acid, and total VFAs in rumen fluid (P<0.0001), rumen fluid pH (P=0.27) or NH_3-N (P=0.70) concentration were influenced by none of treatments. In conclusion, our results demonstrated that although SB and OEO treatments improved rumen VFAs profiles, they had no significant effect on calf performance traits. Based on these results, investigation of the effects of using these treatments as a strategy in rumen development and early weaning of calves is suggestable.

KEY WORDS blood metabolites, fermentation parameters, Holstein suckling calves, oregano essential oil, performance characteristics, sodium butyrate.

INTRODUCTION

Calf breeding is an essential part of dairy farm management because it is one of the herd's most profitable sources of income. Approximately 20 to 30% of dairy cows are culled annually for various reasons (Mahjoubi *et al.* 2020). To prevent a decline in milk production, at least equal numbers of heifers must be introduced into the herd. In conception to weaning, a calf's life is significantly perilous (Seifzadeh *et al.* 2017). The development of the gastrointestinal (GI) tract, especially the rumen, is one of the most important steps profoundly affecting the nutritional status and growth performance of young dairy calves and lactation performance during their adult lives (Liu *et al.* 2021). Feed addi-

tives have been investigated as a means of promoting the growth and health of weaned calves (Gading *et al*. 2020). Although antibiotics have been shown to improve rumen development, increase growth performance, decrease diarrhea prevalence, and reduce mortality in calves (Poudel *et al*. 2019), many countries have banned their use due to bacterial antibiotic resistance, antibiotic residue in animal products, and digestive problems. Therefore, finding an appropriate alternative to antibiotics has recently been a top priority for researchers (Shehta *et al*. 2019).

Antimicrobial medicinal plants and bioactive compounds in their extracts and essential oils can increase digestive secretions, regulate blood circulation, exert antioxidant activity, bolster the immune system, and enhance growth performance (Irwan *et al*. 2021). These compounds have been investigated as potential antibiotic substitutes (Vakili *et al*. 2013). It has been suggested that essential oils as natural additives improve palatability, stimulate appetite and salivary secretion and advance the development of rumen papillae. During the suckling period, the stimulation of appetite and the improvement of digestion and nutrient absorption result in faster growth, earlier weaning, and lower calf rearing costs (Tapki *et al*. 2020).

Oregano generates secondary metabolites, such as essential oils containing effective substances such as carvacrol. Oregano essential oil (OEO) has broad antimicrobial activity, particularly against gram-positive bacteria, by disrupting the bacterial cell membrane that can lead to improved nutrient digestion (Benchaar and Greathead, 2011). Synthetic antioxidants can be replaced by OEO because strong antimicrobial and antioxidant activities are present due to high carvacrol or thymol concentrations (Paraskevakis, 2018). Oregano and thyme essential oils increased suckling calves' growth performance and feed intake (Seirafy and Sobhnirad, 2017).

Butyrate and other short-chain fatty acids (SCFAs) have been proposed as another group of antibiotic alternatives. There is increasing research interest in using these additives as growth promoters in animal production. Recent research indicates that butyrate, a SCFA produced during anaerobic fermentation in the digestive tracts of ruminants, is an effective calf feed additive. Adding this compound to starter feeds or milk replacers improved the performance, health, and glucose metabolism of newborn calves (Araujo *et al*. 2015). Sodium butyrate (SB) and calcium butyrate are the most useful forms of butyrate because they are more stable and palatable (Davarmanesh *et al*. 2015; Liu *et al*. 2021).

SB was shown to be an important regulator and stimulator of epithelial cell proliferation, differentiation, and apoptosis in the stomach and small intestine of calves and piglets (Guilloteau *et al*. 2009). Addition of SB in milk replacer stimulated pancreatic secretion, villus growth, and brush border and pancreatic enzyme activity, which resulted in improved digestibility and better performance and health of calves (Guilloteau *et al*. 2010). In pre-ruminant calves, infusion of butyrate directly into the lumen of the developing rumen stimulated ruminal epithelial cells proliferation and reduced their apoptosis. This resulted in longer rumen papillae and, in consequence, most likely a larger surface area for nutrient absorption, as shown in growing but already ruminating or mature ruminants (Malhi *et al*. 2013).

In addition, other studies reported a reduction in diarrhea and an improvement in health status following the addition of butyrate to milk substitutes (Araujo *et al*. 2015). Guilloteau *et al*. (2009) reported that supplementation of a milk replacer with SB enhanced the growth performance of young calves held under practical farm conditions. SB supplements enhance nutritional performance and accelerate weaning in calves (Kotunia *et al*. 2004). Gorka *et al*. (2009) demonstrated that adding SB to starter feed and milk replacers simultaneously stimulates and develops the rumen and lower digestive tract. As a result, the performance of calves is improved.

According to the mode of action of SB and OEO, it seems that the simultaneous use of these two treatments has synergistic effects. There were no scientific studies on using combination of essential oils and butyrate salts. Consequently, the current study was conducted to determine the effects of SB and OEO on performance traits and certain blood and fermentation parameters in weaned Holstein calves.

MATERIALS AND METHODS

This research was conducted in a 3-month period from December to February at the Taliseh Nemooneh Dairy Farm in Shahryar, Tehran Province, Iran. 24 newborn calves weighing 36 ± 2 kg were randomly assigned to one of the experimental treatments. They were housed in individual pens with a separate feeder and water bowl. Each calf received 2 L of colostrum in two meals, the first given immediately after birth and the second 6 hours apart. The colostrum was fed for an additional two days based on a 10% body weight increase. During the weaning period, the calves were given two meals of 3 L milk daily at 7:00 A.M. and 4:00 P.M. They had unrestricted access to drinking water. The experimental diets were consisted of: 1- a basal diet (control, no additives), 2- basal diet + 5 g SB, 3- basal diet + 5 g OEO, and 4- basal diet + 5 g SB and 5 g OEO. The calves were given a milk solution containing SB, and OEO. The basal diet was consisted of the same ingredients as the starter used in animal husbandry, which is listed in Table 1. Javane Khorasan Co and Barij Essence Pharmaceutical Co,

respectively, supplied SB (Sigma-Aldrich, CAS-Number: 156-54-7) and OEO (carvacrol: 60.55%; thymol: 4.09%; γ-terpinene: 3.05%).

Table 1 Ingredients and chemical composition of starter diet

Ingredients (%)	% DM
Barley grain	40.00
Ground corn grain	22.80
Soybean meal	34.20
Dicalcium phosphate	0.50
Mineral-vitamin premix[1]	1.00
Salt	0.50
Oyster powder	1.00
Chemical composition	**% DM**
DM	91.24
CP	21.32
Ash	6.60
Ca	0.52
P	0.53
NDF	19.23
ADF	7.36
ME (Mcal/kg)	3.05
NE_g (Mcal/kg)	1.34
NE_m (Mcal/kg)	2.08

[1] Mineral-vitamin premix composition (per kg): vitamin A: 10000000 IU; vitamin D3: 150000 IU; vitamin E: 2000 IU; Antioxidant: 0.4 g; Sodium bicarbonate: 71 g; Magnesium sulfate: 19 g; Ferrous sulfate: 3 g; Manganese oxide: 2 g; Zinc sulfate: 3 g; Copper sulfate: 0.3 g and Calcium sulfate: 0.1 g. DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ME: metabolisable energy; NE_g: net energy for gain; NE_m: net energy for maintenance.

Feed intake was obtained from the difference between the feed offered and feed refusal. Calves were weighed on a digital scale every two weeks. Body length, heart girth, withers height, hip height, and hip width were measured using a caliper and tape measure at 14, 28, 42, and 56 days of age (Bayatkouhsar *et al.* 2013). On the 7th, 21st, 42nd, and 56th days of the experiment, four hours after feeding, blood samples were collected intravenously and via the jugular vein. Before analysis, blood samples were centrifuged (3500 rpm) and stored at -20 ˚C. Blood parameters, including glucose, albumin, total protein, β-hydroxybutyrate (BHBA), total cholesterol, triglycerides, and urea concentrations were measured using a commercial kit (Pars Azmun Co).

On the 21st, 42nd, and 56th days of the experiment, rumen fluid was collected using a vacuum pump and stomach tube four hours after morning feeding. In an attempt to minimize errors because of salivary contamination, samples (~500 ml) were collected after discarding an initial 500 mL aliquot of the rumen fluid. Immediately after ruminal fluid sampling, pH was measured using a digital pH meter. (WTW pH meter inoLab 7310 P). To determine the ammonia nitrogen (NH_3-N) concentration, 5 mL of rumen fluid was mixed with 5 mL of 0.2 normal hydrochloric acids. Samples were kept frozen at -21 ˚C until analysis.

The concentration of NH_3-N was determined through the phenol hypochlorite method and via spectrophotometry (Shimadzu Plus3600-UV, Broderick and Kang, 1980). For the determination of volatile fatty acids (VFAs), a 2 mL sample was placed in centrifugal tubes, mixed uniformly with 0.5 mL of 25% or thophosphoric acid, and then centrifuged at 10000 rpm for 10 min. The supernatant was analyzed using gas chromatography (GC8A, Shimadzu Corp., Kyoto, Japan). The temperature of the injector/detector and the column were 260 ˚C and 220 ˚C, respectively (Mao *et al.* 2017)

The performance and VFAs data analysis was done using completely randomized design (CRD). The statistical model was as below:

$$Y_{ij}= \mu + T_i + e_{ij}$$

Where:
Y_{ij}: amount of each observation.
μ: total mean.
T_i: treatment effect.
e_{ij}: random residual error.

Data processing was done using ANOVA process of SAS (9.1) software. Means were compared using the least significant difference test (LSD), at a significant level of $P<0.05$ (SAS, 2003). The initial body weight was considered as a covariate factor in the statistical model. After analysis, this factor was removed due to it being insignificant.

The repeated-measures design and the MIXED procedure were used for the data analysis during the period (blood parameters, and pH and NH_3-N concentration of ruminal fluid). The Tukey method was used to compare the means with a significance level of 5%. The design utilized the following statistical model:

$$Y_{ijk}= \mu + T_i + P_j + TP_{ij} + e_{ijk}$$

Where:
Y_{ijk}: amount of each observation.
μ: mean effect.
T_i: treatment effect.
P_j: time effect.
TP_{ij}: interaction effect of period and treatment.
e_{ijk}: experimental error.

RESULTS AND DISCUSSION

Table 2 demonstrates the effects of OEO and SB on the performance traits of calves. Daily and total feed intake were identical across all treatments. However, calves re-

ceiving the mixture of OEO and SB exhibited an increasing trend (P=0.06) when compared to the control, OEO and SB. The intake of calf starter in young calves is crucial because it determines the animal's health and growth after weaning (Eskandari *et al.* 2021). Kristensen *et al.* (2007) reported that a higher feed intake in the first days after birth resulted in a higher feed intake at weaning.

Because feed intake is influenced by numerous factors, such as body condition, management, climate, diet ingredients, microbial population compatibility, rumen propionate production, physical nutrient composition, digestibility, and feed agronomic characteristics, contradictory results have been reported regarding how OEO affects feed intake (Oba and Allen, 2003; Liu *et al.* 2020). Seirafy and Sobhanirad (2017) and Wu *et al.* (2020) observed that the addition of OEO to milk (5 mL/day. calf) increased starter intake and total feed intake. Increased intake of dry matter (DM) by calves has been linked to volatile and aromatic compounds in essential oils (Liu *et al.* 2020). Phytogenic compounds or essential oils have been found to improve feed digestibility and feed intake by stimulating sensory receptors of the nose and mouth and increasing digestive secretions, which in turn improve feed digestibility and feed intake (Safari *et al.* 2016).

Inconsistent evidence exists concerning the effects of adding butyric acid to milk replacer on starter consumption. This method of compound consumption (added to whole milk, milk replacer, or solid feed) appears effective in this respect (Niwińska *et al.* 2017). Similar to our findings, Liu *et al.* (2021), Hill *et al.* (2007), Vazquez-Mendoza *et al.* (2020), and Ferreira and Bittar (2011) observed that SB supplementation did not affect the intake of dry feed by suckling calves. According to Mahjoubi *et al.* (2020), however, SB supplementation of whole milk increased starter intake. Studies had also reported a decrease in starter intake when SB was added to milk replacers (Wanat *et al.* 2015; Sun *et al.* 2019).

In the current study, daily and total weight gain was numerically higher in the SB and OEO + SB treatment compared to the control (P=0.11). SB supplementation in calf milk replacer has been shown to predominantly affect small intestine and pancreas development and function. It increased mitotic and decreased apoptotic index in the jejunal epithelium, and longer intestinal villi and thicker tunica mucosa were observed in the duodenum, and proximal and distal jejunum. Butyrate addition into milk replacer also increased the activity of main brush border enzymes and the secretion of pancreatic juice as well as chymotrypsin and lipase (Guilloteau *et al.* 2009; Górka *et al.* 2011). OEO has broad antimicrobial activity, particularly against gram-positive bacteria, by disrupting the bacterial cell membrane that can lead to improved nutrient digestion (Paraskevakis, 2018). Therefore, an increase in animal performance due to SB and OEO supplementation is expected. Liu *et al.* (2021) examined the weight gain of calves fed 15, 30, and 45 g SB/day in milk or milk replacer and discovered a positive trend toward improvement compared to the control. Guilloteau *et al.* (2009) reported that replacing milk with 3 g of SB/kg DM improved the feed conversion ratio (FCR) and increased calf weight. Several studies have reported different modes of action of SB supplementation in young animals. One study suggested that butyrate might enhance growth performance in young calves by improving feed digestibility (Beharka *et al.* 1998), while another study reported that butyrate enhanced the absorption capacity of nutrients by increasing the depth of the crypts and the length of small intestine villi, thus increasing the absorptive surface area, in rats and pigs (McCurdy *et al.* 2019). In newborn calves, it has been reported that SB stimulatesthe development of the rumen (Gorka *et al.* 2011) and small intestines (Gorka *et al.* 2014) and enhance the maturation of the intestinal tract (including increased villus size and activities of digestive enzymes) (Guilloteau *et al.* 2009). According to Guilloteau *et al.* (2010), oral butyrate supplementation at a low dose (0.3% of DM intake) increased digestibility of some components (fat, ash, Ca) in calves fed a milk formula based on soybean protein. However, Kato *et al.* (2011) reported that adding SB to milk at concentrations of 3, 5, and 7 g/day did not affect calf weight gain. Different dietary components, types, and amounts of butyrate supplements may produce varying outcomes (Gorka *et al.* 2011).

According to Seirafy and Sobhanirad (2007), also revealed that OEO did not affect body weight and daily weight gain, but a starter and total feed intake were higher, which is consistent with this study. In a study, thyme, rosemary, and oregano essential oils were added to calf diets before and after weaning, increasing daily weight and younger weaned calves (Jeshari *et al.* 2016). Calf performance is enhanced when essential oils are applied in the correct concentration (Vakili *et al.* 2013). Consequently, the insignificant effect of OEO on calf weight gain can be attributed to the concentration employed in this study.

Treatments did not affect the FCR (P=0.45). This result agreed with several studies (Kato *et al.* 2011) and disagreed with others (Guilloteau *et al.* 2009; Serbester *et al.* 2014). This discrepancy can be explained by the type of salt used (calcium or sodium) and the method of consumption. For instance, Davarmanesh *et al.* (2015) added calcium salt to the milk replacer for 21 days, and then they added it to the starter. In this context, the quantity consumed also appears to be crucial. Hill *et al.* (2007), for example, substituted SB for 3% of milk's DM. In contrast, Kato *et al.* (2011) utilized higher doses ranging from 3 to 7 g.

Similarly, Seirafy and Sobhanirad (2017) and Chaves *et al.* (2008) observed that OEO did not affect the FCR of suckling calves and lambs. In contrast, Tapki *et al.* (2020) found that OEO treatment improved the FCR in suckling Holstein calves. Different animal species or forms of oregano oil may account for these contradictory findings.

The effects of OEO, SB and their combination on skeletal growth indices are shown in Table 3. The birth body length of calves treated with OEO and SB was shorter (P=0.007) than that of control. The withers height (P=0.075) and hip height (P=0.028) of animals treated with OEO were also lower than those of the control group. In addition, at 14 days of age, the heart girth of animals treated with SB was smaller (P=0.054) than that of control animals.

In general, the treatments with SB, OEO, and their combination compensated for the disparities above. In all treatments, withers and hip heights on day 14 of the experiment and body length on day 42 were identical. Furthermore, heart girth was the same in all treatments on day 28, and it was greater (P=0.055) in the SB and combination treatments than in the control group on day 56.

According to Beiranvand *et al.* (2014) and Ferreira and Bittar (2011), the dietary supplements of SB, sodium propionate, and calcium propionate did not affect the growth and performance parameters of nursing calves. One study found that supplementation with SB increased wither height in lambs (Heinrichs *et al.* 2007). There is evidence that the addition of feed additives to the diets of Holstein dairy calves had no effect on skeletal development (heart girth and withers height) (Mohamadi Roodposhti and Dbiri, 2012). Similarly, Saremi *et al.* (2004) observed that the use of yeast did not affect the body length, pin width, hip width, pin-to-hock length, or leg size of Holstein dairy calves.

The effects of OEO, SB, and their combination on blood parameters of calves are presented in Table 4. Except for total protein (P=0.002), none of parameters were affected by the treatments. The control treatment resulted in a blood total protein concentration of 15.41 g/dL, which was lower by OEO, SB, and their combination. Blood sampling time was effective on the glucose (P=0.0001), total cholesterol (P=0.02), albumin (P=0.0001), and BHBA (P=0.0001) levels. The blood glucose level decreased with increasing age. Conversely, the levels of total cholesterol, albumin, and BHBA rose as the age of the calves increased. The interaction effect of sampling time and treatment was not significant.

Consistent with these findings, Selvi and Tapki (2019) found that adding OEO to milk replacer did not affect blood glucose, triglyceride, or cholesterol levels in Holstein's calves. In a study by Shahabi and Chashnidel (2012), the administration of a 2 g/kg OEO diet to Dallagh lambs had no effect on their blood glucose and triglyceride levels.

The present study concurs with Seirafy and Sobhanirad (2017) findings that OEO added to milk (5 mL/day.calf) decreased total triglycerides and total cholesterol in suckling calves, whereas blood urea nitrogen increased, which was contrary to our findings. According to Timas *et al.* (2019) and Abdi-Benemar *et al.* (2020), also indicated that adding 5 g of SB to the starter, did not affect the plasma cholesterol and triglyceride concentrations of suckling calves.

Glucose is considered as the preferred energy substrate in pre-ruminant calves (Donkin and Armentano, 1995). This study found no effect of SB supplementation on blood glucose levels, which is consistent with the findings of Ghaffari *et al.* (2021), McCurdy *et al.* (2019), and Ślusarczyk *et al.* (2010). In contrast, Gorka *et al.* (2011) and Nazari *et al.* (2012) reported that adding butyric acid significantly increased plasma glucose concentration. The increased plasma glucose concentration in calves administered SB may be attributable to the inhibition of glucose oxidation in intestinal mucosal cells by butyric acid on glucose oxidation pathways, particularly by inhibition of pyruvate oxidation (Davarmanesh *et al.* 2015). SB supplementation boosted gluconeogenesis as well (intravenous SB supplementation). Nevertheless, Kato *et al.* (2011) and Frieten *et al.* (2018) observed that butyric acid decreased plasma glucose concentration by enhancing insulin sensitivity. This discrepancy may be due to the length of the study or the quantity and method of SB administration (Eskandari *et al.* 2021).

BHBA concentration is a rumen development index in suckling calves, and its increase indicates improved rumen activity and development. It also positively relates to the intake of starter by calves. Mahjoubi *et al.* (2020) reported an increase in BHBA concentration in calves fed 4 or 8 g of SB/day (added to milk). Timas *et al.* (2019) and Abdi-Benemar *et al.* (2020) also found that supplementing suckling calves with SB increased their blood BHBA concentration. However, results of in this study, failed to show SB effect on the concentration of BHBA.

Similar findings, had been reported in several studies (Ferreira and Bittar, 2011; Araujo *et al.* 2015). This result may be explained by the fact that this compound could pass into the small intestine, exerting its beneficial effects on intestinal mucosa development and intestinal enzyme activity (Eskandari *et al.* 2021). It is believed that BHBA is not detectable in the peripheral blood of SB-fed calves because it is metabolized in the digestive tract and liver. In other words, butyrate is likely used directly as an energy source by cell membranes in the digestive tract, thereby enhancing animal performance (Gorka *et al.* 2011).

Table 2 Effect of oregano essential oil and sodium butyrate on performance traits (kg) of suckling calves

Item	Treatments				SEM	P-value
	Control	OEO	SB	OEO+SB		
Initial weight	35.83	32.11	36.83	36.00	2.11	0.43
Final weight	62.50	61.83	67.32	70.33	3.38	0.26
Total weight gain	26.67	29.67	30.50	34.33	2.67	0.11
Daily weight gain (g)	476.19	529.76	544.64	613.10	47.68	0.11
Total feed intake	67.73	67.79	71.50	78.74	3.04	0.06
Daily feed intake (g)	1209.50	1210.63	1276.76	1406.23	54.26	0.06
Feed conversion ratio	2.66	2.29	2.41	2.36	0.17	0.45

OEO: oregano essential oil and SB: sodium butyrate.
SEM: standard error of the means.

Table 3 Effect of oregano essential oil and sodium butyrate on skeletal growth indices (cm) of suckling calves

Item	The day after birth	Treatments				SEM	P-value
		Control	OEO	SB	OEO+SB		
Body length	Initial	37.83^{a}	34.67^{c}	36.10^{bc}	36.83^{ab}	0.575	0.007
	14	38.00^{a}	35.33^{c}	36.40^{bc}	37.67^{ab}	0.515	0.005
	28	38.83^{a}	38.33^{ab}	38.40^{ab}	37.83^{b}	0.298	0.165
	42	41.42	41.75	41.10	42.20	0.393	0.231
	56	43.83	45.08	44.20	44.25	0.462	0.301
Withers height	Initial	75.58^{a}	73.92^{b}	75.00^{ab}	75.33^{a}	0.453	0.075
	14	77.00	77.50	77.40	77.25	0.602	0.941
	28	80.00	82.50	82.20	80.00	1.147	0.270
	42	82.33	85.00	86.20	82.83	0.200	1.399
	56	84.17	87.50	87.80	84.00	1.640	0.220
Hip height	Initial	74.00^{a}	72.00^{b}	72.90^{ab}	73.67^{a}	0.461	0.028
	14	75.67	75.33	75.20	75.00	0.527	0.838
	28	77.83^{ab}	80.00	79.80^{a}	76.17^{b}	1.199	0.110
	42	80.67^{b}	83.50^{ab}	85.20^{a}	80.17^{b}	1.314	0.050
	56	82.00^{ab}	85.00^{a}	85.40^{a}	80.67^{b}	1.449	0.087
Hip width	Initial	12.00	12.00	12.00	12.50	0.478	0.844
	14	13.17	13.33	13.60	14.00	0.440	0.575
	28	16.50^{ab}	16.50^{ab}	15.80^{b}	17.50^{a}	0.380	0.038
	42	19.83	19.33	20.20	20.33	0.345	0.203
	56	22.83	23.00	23.80	23.67	0.474	0.403
Heart girth	Initial	72.67	72.17	72.60	67.67	3.120	0.623
	14	76.83^{a}	73.83^{a}	74.40^{b}	75.83^{ab}	0.783	0.054
	28	87.50	76.67	76.20	77.00	0.845	0.276
	42	93.33	96.00	96.60	94.32	1.896	0.222
	56	97.00^{b}	100.67^{ab}	102.40^{a}	101.50^{a}	1.372	0.055

OEO: oregano essential oil and SB: sodium butyrate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Sun *et al.* (2019) found that plasma urea concentrations of calves fed SB were lower than those of calves fed acidified milk. They asserted that SB promotes a healthy balance of amino acids, thereby increasing protein synthesis and utilization. The increased total protein concentration in calves fed SB can be attributed to the supplement's positive effects. This can sequel to the improved protein accessibility of developing organs. In the present study, however, SB did not affect total plasma protein content.

Similar findings were reported by Ghaffari *et al.* (2021), Sun *et al.* (2019), Guilloteau *et al.* (2010), and Timas *et al.* (2019) regarding the effect of SB supplementation on the total protein and albumin concentration of calves.

According to our findings, the time of blood collection affected the concentrations of glucose and BHBA in the blood. The blood glucose concentration was higher in the first blood sampling than second.

BHBA concentration, however, increased with age. Regardless of diet, it appears that as the rumen and digestive system develop in ruminants, glucose concentration decreases and BHBA concentration increases. This result was validated in weaned calves (Quigley, 1996). In young cattle, BHBA is commonly used to indicate rumen metabolic development (Bergman, 1990). Several studies have found that blood BHBA concentrations increase with calf age (Ślusarczyk *et al.* 2010).

Table 4 Effect of oregano essential oil and sodium butyrate on blood parameters of suckling calves

	Blood metabolites						
	Glu (mg/dL)	TG (mg/dL)	TC (mg/dL)	TP (g/dL)	Alb (g/dL)	BUN (mg/dL)	BHBA (mmol/L)
Treatments							
Control	87.87	36.87	102.38	15.41[a]	3.45	27.00	0.24
OEO	107.25	45.75	114.25	7.29[b]	3.52	22.00	0.18
SB	99.5	29.87	114.25	7.42[b]	3.61	24.25	0.20
OEO+SB	99.00	33.37	110.37	7.70[b]	3.59	25.12	0.19
SEM	8.49	6.55	8.88	0.63	0.12	3.46	0.03
P-value	0.45	0.45	0.76	0.002	0.78	0.79	0.57
Time							
7	123.88[a]	45.75	88.00[b]	7.81	3.25[c]	26.62	0.11[b]
21	91.87[b]	34.37	120.63[a]	7.16	3.44[b]	26.87	0.16[b]
42	94.50[b]	37.25	127.00[a]	11.92	3.72[a]	23.25	0.25[a]
56	67.12[c]	28.50	125.63[a]	10.92	3.76[a]	21.62	0.31a
SEM	6.53	7.23	8.58	1.41	0.08	2.66	0.02
P-value	0.0001	0.44	0.02	0.12	0.0001	0.33	0.0001

Glu: glucose; TG: triglyceride; TC: total cholesterol; TP: total protein; Alb: albumin; BUN: blood urea nitrogen and BHBA: beta hydroxybutyrate.
OEO: oregano essential oil and SB: sodium butyrate.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

They proposed that Ślusarczyk *et al.* (2010), transition from a liquid to a solid diet indicates a change in the sources of physiological fuel.

According to Quigley and Bernard (1992), after 28 days, the concentration of BHBA in calf blood increased from 0.22 mmol to 0.62 mmol. On the other hand, Coverdale *et al.* (2004) found no correlation between animal age and BHBA concentration.

The developmental parameters of the rumen papilla are negatively correlated with blood glucose concentration (Manzanilla *et al.* 2006), which is dependent on the calf's age and the type and quantity of feed consumed. As the rumen begins to develop, blood glucose levels decrease due to the reduction in hyperglycemia brought on by the cessation of milk consumption and the change in carbohydrate availability caused by fermentation in the rumen (Fahey and Berger, 1998). As a calf age, the blood glucose concentration decreases, indicating a decrease in glucose oxidation in ruminal mucosa cells. Due to microbial fermentation, the mitochondria of mucosal cells utilize VFAs more frequently.

Consistent with the present study, Ślusarczyk *et al.* (2010) found that their blood glucose concentration decreased as calves grew. Higher blood albumin concentration may indicate a higher intake of milk protein and solid feed, as these metabolites transport vitamins, minerals, unsaturated fats, hormones, and other beneficial compounds throughout the immune system and function as antioxidants. The immune system is strengthened by increasing blood albumin concentration (Hosseinabadi *et al.* 2013).

Table 5 displays the effect of SB, OEO, and their combination on VFAs concentration in the rumen. The treatments were found to differ significantly (P<0.0001).

The concentrations of acetate, propionate and butyrate were greater in the treatments with OEO and SB than in the control treatment and the combination of OEO with SB. In addition, OEO and SB demonstrated significant treatment differences. In the OEO and SB treatments, iso-butyrate concentrations were lower than in the control group. Valerate decreased in the OEO + SB treatment compared to the control. Also, treatments reduced the concentration of isovalerate. The acetate-to-propionate ratio was unaffected by treatment with OEO or a combination of OEO and SB. Treatments of SB and OEO increased the total VFAs compared to the control. Calves' rumen fermentation starts at very young age and VFAs can be detected as early as two weeks after birth (Beharka *et al.* 1998). The byproducts of rumen fermentation are acetate, propionate, and butyrate. The greater the rate of fermentation, the greater the concentration of VFAs. It has been shown that treatment of OEO and SB combination resulted in a desirable increase in butyrate concentration (Poudel *et al.* 2019). The correlation between blood glucose concentration and propionate production is positive. Boosting performance and weight gain by increasing the molar volume of propionic acid absorbed in the rumen and converted to glucose in the liver had been demonstrated. More than 50% of ruminant blood glucose is produced in the liver via gluconeogenesis of propionate. In the present study, calves fed OEO, and SB showed a significant increase in concentration of propionate, which may explain the numerically greater blood glucose concentration in these treatments (Huntington *et al.* 2006).

According to Shahi *et al.* (2015), using 600, 800, and 1000 mg/day of lime essential oil increased propionate levels and decreased butyrate levels and acetate-to-propionate ratios in male Holstein calves.

Table 5 Effect of oregano essential oil and sodium butyrate on rumen fluid volatile fatty acid concentration (mmol/L) of suckling calves

Item	Treatments				SEM	P-value
	Control	OEO	SB	OEO+SB		
Acetate	19.20^{c}	32.09^{b}	44.94^{a}	18.94^{c}	0.351	< 0.0001
Propionate	12.65^{c}	21.97^{a}	18.27^{b}	11.72^{c}	0.743	< 0.0001
Butyrate	1.91^{c}	3.68^{b}	4.21^{a}	2.07^{c}	0.084	< 0.0001
Iso-butyrate	0.49^{a}	0.34^{b}	0.36^{b}	0.48^{a}	0.015	< 0.0001
Valerate	1.19^{ab}	1.16^{b}	1.35^{a}	0.59^{c}	0.057	< 0.0001
Iso-valerate	0.57^{a}	0.35^{c}	0.32^{c}	0.47^{b}	0.020	< 0.0001
Acetate/propionate	1.52^{b}	1.46^{b}	2.53^{a}	1.75^{b}	0.114	< 0.0001
Total VFA	36.00^{c}	59.58^{b}	69.45^{a}	30.43^{d}	1.196	< 0.0001

OEO: oregano essential oil and SB: sodium butyrate.
VFA: volatile fatty acids.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In contrast, using 250 or 500 mL of OEO/mL of rumen fluid did not affect the TVFAs, acetate, and propionate concentration, according to an *in vitro* study by Yadeghari *et al.* (2015), however, at 750 and 500 mg/mL level, the concentration of butyrate increased. Adding commercial essential oil blends to milk replacers for suckling Holstein calves increased their production of VFAs (Poudel *et al.* 2019).

In an *in vitro* continuous culture system and at a constant pH, Castillejous *et al.* (2005) observed that adding 1.5 mg of a blend of plant essential oils increased their overall production without affecting the proportion of individual VFAs. In a similar study conducted by Castillejous *et al.* (2007), adding 5 mg/L of herbal essential oil blend increased TVFAs and acetic acid production. Several *in vitro* continuous culture studies have demonstrated that plant essential oils influence the pattern of VFAs production in the rumen as a function of pH and diet (Cardozo *et al.* 2005).

Cinnamaldehyde and pepper, for instance, increased the ratio of acetate to propionate at pH 7. At pH 5, however, this ratio decreased. Studies demonstrate that SB supplements improves rumen papillae development and growth (Gorka *et al.* 2011; Koch *et al.* 2019). The ideal supplementation level of SB is essential for rumen development and VFAs concentration. Soltani *et al.* (2017) study showed that supplementation with SB altered the concentration of SCFAs. Therefore, SB supplementation affected the molar ratio of acetate and butyrate both negatively and positively. Liu *et al.* (2021) reported that SB supplementation did not affect total or individual ruminal VFAs concentrations.

For growth, cellulolytic bacteria's rumen and structural carbohydrates require sufficient amounts of branched chain fatty acids (BCFAs). Including iso-butyrate and iso-valerate in milk and starter promotes cellulolytic bacteria, which enhances fermentation and rumen enzyme activity, thereby boosting calf growth (Diao *et al.* 2019).

Although the concentrations of acetate, propionate, and butyrate were higher in the OEO and SB treatments compared to the control group, the concentrations of iso-butyrate and iso-valerate decreased. This result may explain why the treatments did not improve animal performance.

According to the results of the mean comparison (Table 6), OEO, SB, and their combination did not affect the NH_3-N concentration (P=0.31) or pH (P=0.27) in the rumen fluid of calves. The interaction effect of sampling time and treatment was not significant.

Table 6 Effect of oregano essential oil and sodium butyrate on rumen fluid ammonia nitrogen (mg/dL) and pH of suckling calves

	Item	
	pH	NH_3-N
Treatments		
Control	6.32	6.51
OEO	6.48	6.90
SB	7.11	7.76
OEO + SB	6.11	6.28
SEM	0.31	0.50
P-value	0.27	0.31
Time		
21	6.95	11.24^{a}
42	6.92	6.17^{b}
56	6.85	2.11^{c}
SEM	0.31	0.15
P-value	0.82	P < 0.001

OEO: oregano essential oil and SB: sodium butyrate.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Several studies have revealed that the effects of plant essential oils on fermentation parameters such as NH_3-N and pH are highly variable. These variations are attributable to variables such as the type and origin of the essential oil, its chemical composition, dosage, antagonistic interactions between the active compounds in the essential oil and the diet, and experimental conditions (Benchaar *et al.* 2008).

In some *in vitro* studies, a reduction in rumen fluid nitrogen concentration was observed (Castillejous *et al.* 2006). However, most *in vivo* studies indicate that essential plant oils do not affect this parameter (Yang *et al.* 2010). In a study by Akbarian-Tefaghi *et al.* (2018), the rumen fluidNH_3-N content of suckling calves before and after weaning was found to be unaffected by the essential oils used in the starter diet.

In line with our findings, Liu *et al.* (2021) reported that feeding 15, 30, or 45 g/day SB did not affect the rumen nitrogen concentration of calves. They explained that this is due to rumen microbes' balance between protein degradation and NH_3-N absorption. In addition, it was observed that SB treatment did not affect the rumen fluid's pH. This phenomenon is attributable to the passage of this supplement along with the liquid feed from the rumen, with no effect on the quantity of DM consumed.

At various sampling times, the pH of rumen fluid remained constant (P=0.82). The amount of NH_3-N in rumen fluid decreased significantly as calves aged due to the effect of time (P<0.0001). This trend of decreasing NH_3-N content may be related to the increased microbial population in the rumen and their increased utilization of available NH_3-N (Bayatkouhsar *et al.* 2013).

CONCLUSION

The addition of OEO and SB to milk replacers had no discernible effect on the performance characteristics of calves, according to our findings. Although SB and OEO did not have synergistic effects, each of these treatments alone improved the production of rumen VFAs. It is recommended to investigate their potential in rumen development and early weaning of calves in future studies.

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