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Does the time of microencapsulated sodium butyrate supplementation have any effect on the growth performance and health of Holstein dairy calves?

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Abstract

The optimal feeding time of microencapsulated sodium butyrate (SB) in whole milk (WM) and starter feed on growth performance and health in dairy calves was evaluated. Forty-eight newborn Holstein calves (body weight (BW) = 39.45 ± 2.48 kg) were randomly assigned to one of the four treatment groups (12 calves per treatment; seven females and five males) in a complete randomized block design and fed (1) WM without microencapsulated SB (CON) supplementation; (2) 4 g/day SB added to WM since days 4–32 (SB-4-32); (3) 4 g/day SB added to WM since days 61–74 and an equal amount was added to starter since days 75–88 (SB-61-88) and (4) 4 g/day SB added to WM since days 4–74 and an equal amount was added to starter since days 75–88 (SB-4-88). Total dry matter intake, starter intake, BW, average daily gain and gain-to-feed were similar between treatments. Calves fed SB-4-32, and SB-4-88 had lower faecal score during pre-weaning, and overall. In addition, calves in SB-4-32 and SB-4-88 groups had fewer numbers of days with scours during the pre-weaning period, and throughout the study. Calves fed SB-61-88 had greater serum total protein during post-weaning. Post-weaning and overall albumin concentrations were greater in SB-4-32 and SB-4-88 calves and tended to be greater in the pre-weaning period compared to control calves. In general, the time of SB addition had no remarkable effect on performance but better faecal score within the pre- and post-weaning periods.

Introduction

Butyric acid, one of the short-chain fatty acids, is a natural substance present in the rumen of ruminants, colons of monogastric species, cow milk (0.16 g/l; Alais, 1984; Guilloteau *et al.*, 2010*a*). It has been well-documented that butyrate is an important stimulator and regulator of ruminal epithelium growth as well as its function. It appears that the indirect effect of sodium butyrate (SB) on rumen development is clearer (Penner *et al.*, 2011). In ruminants, the most important source of butyrate is the microbial fermentation of carbohydrates in the rumen (Bergman, 1990). Within the first 1–2 weeks of a calf's life, the amount of solid feed intake is very low and rumen microflora is not fully functioning; this leads to a very low butyrate concentration in the yet underdeveloped rumen until the regular solid feed intake starts and rumen microflora develops (Anderson *et al.*, 1987; Flaga *et al.*, 2015). Thus, before the development of the rumen, milk butyrate is the main source of this molecule for the newborn calf. On the other hand, calves are fed mostly with whole milk (WM) or milk replacer (MR) before weaning and the abomasum and small intestine are the main sites of feed digestion. Therefore, the development of these gastrointestinal tract (GIT) compartments is crucial for nutrient absorption, performance and health of milk-fed calves. Because GIT development can have impact on the feed intake, the efficiency of digestion and resistance to gastrointestinal disorders and in this way animal growth and health, each method enhancing these processes are highly desirable. Thus, supplementing liquid or starter feed with butyrate may be a good strategy to improve rumen and intestinal development in calves (Gorka *et al.*, 2011*a*).

The effect of dietary butyrate supplementation could be modulated by butyrate protection from its release and utilization in the stomach (both forestomach and abomasum) in order to releasing in the distal sections of the intestine and increasing butyrate content in the large intestine (Mallo *et al.*, 2012). Embedding in the continuous lipid matrix often referred to as microencapsulation or fat coating, is commonly used for this purpose (Claus *et al.*, 2007; Gorka *et al.*, 2011*a*, 2014; Moquet *et al.*, 2016). Of potential advantage, protected butyrate is released slowly from the fat coat, providing the possibility for its more uniform distribution in the small and large intestines. Therefore, protected butyrate is more likely to affect the

structure and function of the large intestine through butyrate delivery to the very last sections of the intestine. This seems to be especially beneficial taking into account the high susceptibility of newborn calves to diarrhoea (Gorka *et al.*, 2018).. It has been well demonstrated in numerous studies that butyrate supplementation through either un-protected or protected SB in MR and starter feed (Hill *et al.*, 2007; Guilloteau *et al.*, 2010*b*; Gorka *et al.*, 2011*a*, 2011*b*, 2014; Roh *et al.*, 2018; Koch *et al.*, 2019; Wu *et al.*, 2022), and acidified milk (Sun *et al.*, 2019) have pronounced effects on growth performance, feed efficiency, GIT development and health of dairy calves through modulation of proliferation, differentiation, stimulated pancreatic secretions and function of the GIT tissues.

Even though butyrate is naturally found in cow's milk, it seems that adding extra SB to milk can improve dairy calves' performance because of its small amount. To our knowledge, there is limited study in neonatal calves in which SB (unprotected form) has been added to WM during the pre-weaning period (Mahjoubi *et al.*, 2020); it has been indicated that SB (4 or 8 g/day) could improve calf performance. Davarmanesh *et al.* (2015) supplemented unprotected form of calcium butyrate salt to WM only for 23 days. On the other hand, only one study indicated the effect of protected butyrate supplementation in MR (Nazari *et al.*, 2012). Also, Mahjoubi *et al.* (2020) stated that the time of SB supplementation might have some impacts on calf performance. Due to the limited documents in this regard, we decided to investigate the different times of SB supplementation to address the above-mentioned concerns.

Based on the early mentioned considerations, we hypothesized that the addition of protected SB to WM and deliver it to the small intestine could improve calf growth performance and health status when it is added in the first month of life and/or in the transition period. Therefore, the aim of the current study was to (1) determine the optimum age for SB inclusion in WM and (2) investigate the effect of WM supplemented with protected SB on the incidence of diarrhoea and calf performance.

Materials and methods

The current experiment was performed from January to April 2021 in a commercial dairy farm (Avin Dasht, Qazvin, Iran) according to the guidelines of the Iranian Council of Animal Care (1995). This farm is located in a sub-tropical area (longitude 49°29′E and latitude 35°57′N).

Calves, treatments and housing

Forty-eight Holstein dairy calves (28 females and 20 males, average body weight (BW) = 39.48 ± 2.48 kg) were randomly assigned to treatments (n = 12 calves per treatment; seven females and five males) in a complete randomized block design. After birth, all calves were separated from their dams immediately and placed in individual pens (1.5 × 2.0 m^2) bedded with clean wheat straw. The calves were fed 4 litres of colostrum at the first 12 h of life (2.5 litres until 1 h after birth and 1.5 litres at 12 h after the first feeding). On days 2 and 3 of life, calves received transition milk (4 litres) in two meals of equal volume (at 8.00 and 20.00 h). After that, calves were randomly assigned into 1–4 experimental treatments from day 4 and were blocked by sex. Treatments were as follows: (1) control without microencapsulated SB (CON) supplementation; (2) 4 g/day SB (Novyrate®C) added to milk since days 4–32 (SB-4-32); (3) 4 g/day SB added to milk since days 61–74 and an equal amount was added to starter since days 75–88 (SB-61-88) and (4) 4 g/day SB added to milk since days 4–74 and an equal amount was added to starter since days 75–88 (SB-4-88). Pre-weaning (from days 4 to 74) the SB was incorporated into WM and mixed by a strew before calf drink and the post-weaning (from days 75 to 88) was top-dressed to starter feed to make sure each calf eats the SB. In order to make sure that each calf consumed the respective amount of SB, it was mixed with ∼50–70 g of daily starter feed allowance and top-dressed. Novyrate®C is a coated butyrate product (containing 320 g SB as microencapsulated, 990 g/kg dry matter (DM), 610 g/kg crude fat, 64 g/kg sodium; Novyrate®C, Innovad Co, Essen, Belgium). Fat coating ensures that butyrate is primarily directed into the small and large intestines where pancreatic lipases gradually remove the fat coating. The gradual releasing pattern of protected SB product was verified by an *in vitro* assessment (https://issuu.com/innovad4/docs/novyratenovbroen28102016ldef). All calves were individually fed the same volumes of WM (35 g fat, 31.5 g crude protein (CP), 46.7 g lactose, 8 g ash and 121.2 g total solids per kg of milk, 3.4 ± 0.7 g/100 g fatty acids butyric acid) two times per day at 8.00 and 20.00 h with an accelerated nutritional plan, which is illustrated in Fig. 1 and weaned at day 74 of age. After weaning, all calves remained in individual stalls until day 88 of age to collect the post-weaning data. The calves had free access to fresh starter feed and water throughout the study (from day 4 until 88). The starter feed composed of 900 g/kg DM concentrate and 100 g/kg DM chopped alfalfa hay was fed as a total mixed ration every morning at 8.30 h. Diet was formulated using National Research Council (2001) software and the chemical composition of the starter feed is presented in Table 1. The starter feed formulation was constant across experimental treatment during the pre- and post-weaning periods.

Measurement of dry matter intake (DMI), BW and health

Throughout the study, offered and refused feed was weighed daily to determine the total starter intake for the individual calf. When the calves consumed more than 1 kg of starter feed for 3 consecutive days, the first day of age that each calf met the specific starter feed consumption target of 1 kg was recorded and used to evaluate the time to consume 1 kg of starter feed. Individual BW (using an electronic scale) and body skeletal growth including body length, withers height, heart girth and hip height were recorded on days 4, 32, 60, 74 and 88 before the morning feeding meal, and average daily gain (ADG) was calculated as the difference between two consecutive BW measurements divided by days. Gain-to-feed ratio was calculated as grams of ADG divided by grams of total DMI (liquid feed DMI + starter feed DMI). Samples of starter feed were collected throughout the study (n = 4, 20 day sampling intervals) for the determination of DM and chemical analyses. Samples of starter feeds were dried in a convection oven (60°C for 48 h). Subsamples of dried feeds were composited by treatment and ground in a mill (Ogaw Seiki Co., Ltd., Tokyo, Japan) to pass a 1-mm screen. Feed samples analysed for CP (AOAC, 2000; 984.13), ether extract (EE; AOAC, 2000; ID 920.39), ash (AOAC, 2000; ID 942.05), neutral detergent fibre (NDF; Van Soest *et al.*, 1991). The alpha-amylase and sodium sulphite were not used in the NDF assay. Milk composition was measured every 14 days from bulk tank samples. An aliquot of milk was frozen (−20°C) without preservatives for subsequent butyric acid analysis. Fatty acid methyl esters of the lipid in milk samples were prepared and then analysed under GC (6890

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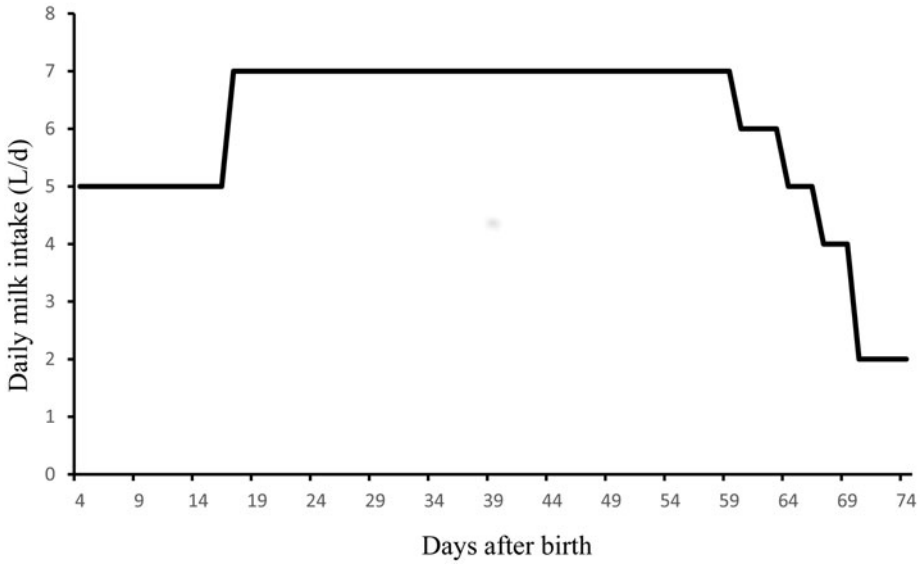

Fig. 1. Schematic diagram represents the amounts of milk consumed (kg/day) by calves; 5 litres of milk/day from 4 to 16 days, 7 litres/day from 17 to 59 days, 6 litres/day from 60 to 63 days, 5 litres/day from 64 to 66 days, 4 litres/day from 67 to 69 days and 2 litres/day from 70 to 74 days of age.

N, Agilent Technologies, Santa Clara, CA, USA) conditions described by Shingfield *et al.* (2003).

Health condition of calves was assessed daily according to Larson *et al.* (1977) and Heinrichs *et al.* (2003). One of the authors performed the health scoring and each time was the same person. Faecal scoring was as follows: 1 = firm, 2 = soft, 3 = soft and running, 4 = watery. General appearance scoring was: 1 = normal and alert; 2 = ears drooped; 3 = head and ears drooped, dull eyes, slightly lethargic; 4 = head and ears drooped, dull eyes, lethargic and 5 = severely lethargic. For calves that need medical treatments, the farm's veterinarian administrated the proper drug, and the treatment was followed according to his recommendation; therefore, medical days, treatment bouts and the number of used drugs were recorded to be statistically analysed.

Table 1. Starter diet ingredients and chemical composition

Item	Contents
Ingredients (g/kg DM)	
Alfalfa, finely chopped	100
Corn grain, ground	558
Soybean meal	270
Vitamin and mineral mix[a]	27
Carbonate calcium	14
Magnesium oxide	5
Sodium bicarbonate	14
Salt	7
Bentonit	5
Chemical composition	
Metabolizable energy[b] (MJ/kg)	12.9
CP (g/kg DM)	180
Non-fibre carbohydrate[c] (g/kg DM)	542
NDF (g/kg DM)	136.8
EE (g/kg DM)	28

[a]Contained per kg of the supplement: 500 000 IU vitamin A, 130 000 IU vitamin D, 6000 IU vitamin E, 10 g Ca, 10 g P, 20 g Mg, 4100 mg Zn, 15 mg Co, 1000 mg Cu, 4000 mg Mn, 35 mg I, 5000 mg Fe and 30 mg Se, 2000 mg monensin.
[b]Calculated using the NRC (2001) model.
[c]Non-fibre carbohydrate was calculated as [DM – (NDF + CP + ether extract + ash)] (NRC, 2001).

Blood sampling and analyses

Blood samples from each calf were collected from the jugular vein into 10 ml tubes 4 h after morning feeding on days 4, 32, 60, 74 and 88. Blood samples were placed on ice immediately after collection and centrifuged at 3000 ***g*** (Kubota Co., Bunkyo City, Tokyo, Japan) for 15 min at 4°C to obtain serum, and then serum samples were frozen at −20°C until future analyses. It took 1 h from blood sampling to storage. Serum subsamples were analysed to determine concentrations of glucose (mg/dl), albumin (g/dl) and total protein (TP, g/dl) using commercial kits (Pars Azmoon Co., Tehran, Iran). Serum concentrations of beta-hydroxybutyrate (BHB, mmol/l) were measured using a commercial kit (Ranbut, Randox Laboratories Limited, Crumlin, County Antrim, Randox, UK); the inter- and intra-assay coefficient of variation for the glucose assay were 2.34 and 2.72%, respectively, and for the BHB assay were 2.91 and 3.45%, severally.

Statistical analysis

Before data analysis, all data were evaluated for normality using the UNIVARIATE procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). A pre-study power analysis for sample size assessment was carried out for the primary response variables, including weight gain, ADG, body skeletal growth and blood metabolites according to recent published literature (Sun *et al.*, 2019; Mahjoubi *et al.*, 2020; Liu *et al.*, 2021; Wu *et al.*, 2022). The predicted sample size was 12 calves per treatment group for variables related to growth performance and blood metabolites ($\alpha = 0.05$ and power = 0.85). Therefore, 48 animals (12 calves per treatment) were considered sufficient to get a significant result with adequate power in performance among the treatment groups. Total DMI, starter feed intake, faecal scores, general appearance scores and blood metabolite data were subjected to analysis of variance (ANOVA) using the MIXED procedure of SAS with time as repeated measures

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during the overall experiment. The model consisted of treatment, sex, time and their interactions were included as the fixed effects, and calf within treatment was included as a random effect. Sex was not significant and thus removed from the original model. Initial BW, initial skeletal growth parameters and blood data were considered as a covariate for the BW, skeletal growth and blood metabolites analysis but were removed from the final models because no differences due to these factors were found ($P > 0.20$). BW, gain-to-feed ratio, body skeletal growth data and days experiencing a health criterion were not analysed as repeated measures and they were analysed by the generalized linear model. Three variance–covariance structures (autoregressive order 1, unstructured or compound symmetry) were tested and the autoregressive order 1 covariance structure yielded the smallest Schwarz's Bayesian information criterion. In addition to the overall F test, differences among treatments were assessed using orthogonal contrast (CON *v.* SB-4-32, SB-61-88 and SB-4-88). The POLYANOVA model was considered for performance and blood data. Linear and quadratic contrasts were reported from this statistical analysis during each period. The statistical model used for analysis was: singledollarY_{ijk} = \mu + {\rm Treatmen}{\rm t}_i + {\rm Tim}{\rm e}_j + \lpar {{\rm Treatment} \times {\rm time}} \rpar _{ij} + {\rm cal}{\rm f}_k + \beta \lpar {Xi-X} \rpar + e_{ijk}singledollarwhere Y_{ijk} is the dependent variable, μ is the overall mean, Treatment_i is the fixed effect of treatment, Time_j is the fixed effect of time, $(\text{Treatment} \times \text{Time})_{ij}$ is the fixed effect of interaction between treatment and time, calf_k is the random effect of calf, $\beta (Xi–X)$ is the covariate variable and e_{ijk} is the residual error.

The number of days with diarrhoea was categorized with a faecal score ≥2 and the general appearance score (1–5) was categorized as the number of days with a general appearance score ≥2 (Jahani-Moghadam *et al.* 2015). Because the variance of the number of days with faecal and general appearance score ≥2 was not uniformly distributed, these variables were square-root transformed for better homogeneity of the distribution of residuals (means shown in Table 5 for these variables are back-transformed). The same was carried out for medical days, treatment bouts and the number of used drugs. The least-squares means for treatment effects was separated by the use of the PDIFF statement. Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

Results

Feed intake and growth performance

The results of DMI, starter intake, BW, ADG and gain-to-feed ratio are given in Tables 2 and 3. In general, daily DMI and starter intake did not differ among experimental treatments at any stage ($P > 0.05$). Supplementation with SB did not have impact on BW, ADG (Fig. 2) and gain-to-feed ratio ($P > 0.05$).

The results of the structural growth indices are given in Table 4. There was no effect of treatments on structural growth indices on different days of the trial ($P > 0.05$).

Health criteria

Faecal scores and general appearance scores are presented in Table 5. Compared to CON and SB-61-88, encapsulated SB inclusion in SB-4-32 and SB-4-88 groups decreased the faecal score during the pre-weaning ($P = 0.043$), and throughout the experiment ($P = 0.034$). However, the general appearance score did not differ among treatments at any stage ($P > 0.05$).

The number of days with loose faecal score (≥2) were lower ($P = 0.035$ and $P = 0.025$; respectively) for calves fed on SB-4-32 and SB-4-88 diets compared to CON and SB-61-88 groups during the pre-weaning period and throughout the experiment; while, did not differ between treatments in the first month of life and the transition period (Table 5). In general, supplementation of the encapsulated SB in WM significantly decreased ($P = 0.039$) the number of days with loose faecal score compared with the control group during the pre-weaning period. With respect the entire period of the study, calves fed SB also tended ($P = 0.066$) to decrease the number of days with loose faecal score compared with the control group. Days with altered general appearance score, medical days, treatment bouts and the number of used drugs did not differ among treatments ($P > 0.05$; Table 5).

Blood metabolites

The results of the blood metabolites are presented in Table 6. The levels of serum glucose were not different among treatments at any stage ($P > 0.05$); however, the glucose concentration was reduced in calves fed SB-61-88 compared with CON and SB-4-88 calves on day 74 ($P < 0.001$). Serum BHB levels were not influenced among experimental treatments during the pre- and post-weaning (day 88) period ($P > 0.05$), but there was a significant effect of the interaction of treatment by time ($P = 0.045$) throughout the study. Serum BHB increased with the advancement in the study, but there was a tendency for SB-4-32 calves to have greater ($P = 0.086$) BHB level than CON and SB-61-88 calves on day 74. There was also a difference at day 88 for SB-61-88 and SB-4-88 calves to have higher ($P < 0.001$) BHB level than CON and SB-4-32 calves. Post-weaning TP concentration was higher ($P < 0.001$) for the SB-61-88 group than for other groups. Serum TP level in the post-weaning period also was significantly more elevated ($P = 0.025$) for encapsulated SB-fed calves than for CON calves. Furthermore, calves fed SB tended ($P = 0.078$) to have higher TP levels compared to CON calves throughout the study. Within the post-weaning and whole period, serum albumin level was higher ($P = 0.010$) in SB-4-32 and SB-4-88 groups than in the CON group. Moreover, calves fed encapsulated SB had higher serum albumin concentrations during the pre-weaning, the post-weaning and overall periods ($P = 0.032$ and $P < 0.001$; respectively) in comparison with calves without SB supplement. In addition, albumin concentration tended to be increased in calves fed SB compared with CON calves on day 60 ($P = 0.067$).

Discussion

Because they are more stable, generally odourless and easier to handle in the feed manufacturing processes, butyrate salts (SB or calcium butyrate) or butyrins (esters of butyrate and glycerol) are often used instead of butyric acid itself in animal studies and in practice (Guilloteau *et al.*, 2010*a*). The SB, the most often used source of dietary butyrate because of its high availability and modest price, dissolves easily in water and rapidly dissociates in water solutions (Mallo *et al.*, 2012).

Feed intake and growth performance

Most research has been conducted on calves in the pre-weaning period by supplementation of butyric acid to formula or solid

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Table 2. Effects of SB supplementation to WM on feed intake of Holstein calves ($n = 12$ calves per treatment)

Item	Treatments[a]				S.E.M.[b]	*P*-value				Contrast
								Time		
	CON	SB-4-32	SB-61-88	SB-4-88		Treat	Treat × time	Linear	Quadratic	CON *v.* SB-4-32, SB-61-88, SB-4-88
Total DMI (g DM/day)										
Days 4–32	810	811	798	813	11.7	0.821	0.034	<0.001	<0.001	0.870
Days 33–60	1194	1169	1114	1161	48.5	0.713	0.642	<0.001	0.503	0.413
Days 4–60	1009	995	961	992	32.7	0.773	0.484	<0.001	<0.001	0.480
Days 61–74	1501	1474	1422	1466	107.6	0.966	0.100	<0.001	<0.001	0.707
Pre-weaning[c]	1101	1083	1052	1079	55.5	0.944	0.922	<0.001	0.807	0.648
Transition period[d]	2141	2076	1984	2073	119.7	0.840	0.470	<0.001	<0.001	0.488
Post-weaning[e]	2597	2515	2342	2482	146.1	0.678	0.520	<0.001	0.638	0.374
Overall	1335	1307	1256	1302	70.3	0.891	0.978	<0.001	<0.001	0.569
Starter feed intake (g DM/day)										
Days 4–32	66	67	55	70	11.7	0.821	0.034	<0.001	0.107	0.870
Days 33–60	353	328	272	319	48.5	0.713	0.642	<0.001	0.031	0.413
Days 4–60	216	202	168	199	32.7	0.773	0.484	<0.001	<0.001	0.480
Days 61–74	993	966	914	958	107.6	0.966	0.100	<0.001	<0.001	0.707
Pre-weaning	377	359	328	354	55.5	0.944	0.922	<0.001	<0.001	0.648
Transition period	1915	1850	1758	1847	119.7	0.840	0.470	<0.001	0.188	0.488
Post-weaning	2587	2505	2332	2472	146.1	0.678	0.520	<0.001	0.825	0.374
Overall	717	689	638	685	70.3	0.891	0.978	<0.001	<0.001	0.569
Days to 1 kg/starter intake/day for 3 consecutive days	67	69	68	67	1.7	0.806	–	–	–	0.413

[a]Treatments were: (1) without SB supplement (CON); (2) SB supplement added to milk from 4 to 32 days (SB-4-32); (3) SB supplement added to milk from 61 to 74 days and added to starter from 75 to 88 days (SB-61-88) and (4) SB supplement added to milk (since days 4–74) and the starter (since days 75–88) for the total experiment (SB-4-88).
[b]Standard error of the mean.
[c]Days 4–74.
[d]Days 61–88.
[e]Days 75–88.

feeds. The effect of adding butyric acid to MR on starter consumption has been contradictory (Niwińska *et al.*, 2017). In agreement with previous studies (Hill *et al.*, 2007; Gorka *et al.*, 2011*a*, 2011*b*; Kato *et al.*, 2011; Davarmanesh *et al.*, 2015; Frieten *et al.*, 2017, 2018; Roh *et al.*, 2018; Ghaffari *et al.*, 2021), the addition of SB to WM and MR did not affect DMI and starter intake. Starter consumption is very critical in young calves because it determines their growth and health after weaning (Greenwood *et al.*, 1997). Despite the positive effect of butyric acid in MR on weight gain, Hill *et al.* (2007) found no effect on starter intake, probably because the composition of MR was changed by the addition of butyric acid and the share of whey in replacer powder was reduced. In addition, some studies observed a reduction in starter intake when SB was supplemented with acidified milk and MR (Wanat *et al.*, 2015; Sun *et al.*, 2019). In contrast with the current results, Mahjoubi *et al.* (2020) observed starter intake improvement when SB was supplemented in WM, that this may be using unprotected SB in that study.

The addition of SB to the MR had an impact on small intestine and the growth and function of the pancreas (Gorka *et al.*, 2018); this phenomenon increases the cell division and decreases the cell death index in the jejunum epithelium (Guilloteau *et al.*, 2009*b*; Gorka *et al.*, 2011*b*). In contrast to the current results, the addition of butyric acid to MR or the starter feed has improved (Hill *et al.*, 2007; Guilloteau *et al.*, 2009*b*; Gorka *et al.*, 2011*b*, 2009; Nazari *et al.*, 2012; Liu *et al.*, 2021; Wu *et al.*, 2022) or decreased (Wanat *et al.*, 2015; Ghaffari *et al.*, 2021) calf growth performance. In addition, SB supplementation in WM and acidified milk improved ADG in dairy calves (Sun *et al.*, 2019; Mahjoubi *et al.*, 2020). However, some other researchers have not been observed any effect of inclusion butyric acid in diet on calf performance (Kato *et al.*, 2011; Araujo *et al.*, 2015; Davarmanesh *et al.*, 2015; Frieten *et al.*, 2017, 2018; Roh *et al.*, 2018), which is in line with our results. Some of these differences are due to the type of supplemented butyric acid salt, and others are due to the age of the calf when butyric acid was included in the diet. For instance, Guilloteau *et al.* (2010*a*) observed no significant effect when butyric acid was fed to calves from day 12 after birth. Furthermore, studies show that the greatest effect of butyric acid is in the first week after birth (e.g. Niwińska *et al.*, 2017), which is in contrast to the current study.

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Table 3. Effects of SB supplementation to WM on growth performance of Holstein calves ($n = 12$ calves per treatment)

Item	Treatments[a]				S.E.M.[b]	*P*-value				Contrast
								Time		
	CON	SB-4-32	SB-61-88	SB-4-88		Treat	Treat × time	Linear	Quadratic	CON *v.* SB-4-32, SB-61-88, SB-4-88
BW (kg)										
Day 4 (initial)	39.5	39.7	39.2	39.4	0.74	0.971	–	–	–	0.974
Day 32	51.3	51.7	50.0	50.2	0.83	0.444	–	–	–	0.514
Day 60	69	69	68	70	1.3	0.842	–	–	–	0.783
Day 74 (weaning)	81	81	79	80	1.8	0.904	–	–	–	0.782
Day 88 (final)	95	95	93	95	2.2	0.828	–	–	–	0.745
Overall	1335	1307	1256	1302	70.3	0.891	0.978	<0.001	<0.001	0.569
ADG (g/day)										
Days 4–32	422	435	378	385	30.2	0.454	–	–	–	0.502
Days 33–60	667	642	663	716	32.9	0.417	–	–	–	0.862
Days 4–60	545	539	520	551	24.4	0.829	–	–	–	0.766
Days 61–74	807	852	789	751	59.8	0.670	–	–	–	0.882
Pre-weaning	597	601	574	591	26.2	0.887	–	–	–	0.771
Transition period	953	960	910	945	53.0	0.910	–	–	–	0.811
Post-weaning	1030	1000	965	1072	66.0	0.699	–	–	–	0.813
Overall	670	668	639	671	24.1	0.772	0.501	<0.001	0.704	0.716
Gain-to-feed ratio[f]										
Days 4–32	0.52	0.54	0.47	0.48	0.033	0.394	–	–	–	0.458
Days 33–60	0.57	0.56	0.60	0.62	0.021	0.118	–	–	–	0.277
Days 4–60	0.55	0.55	0.55	0.56	0.019	0.926	–	–	–	0.809
Days 61–74	0.55	0.57	0.57	0.51	0.038	0.572	–	–	–	0.988
Pre-weaning[c]	0.55	0.56	0.55	0.55	0.016	0.964	–	–	–	0.791
Transition period[d]	0.46	0.48	0.48	0.47	0.023	0.916	–	–	–	0.576
Post-weaning[e]	0.39	0.41	0.40	0.42	0.025	0.827	–	–	–	0.479
Overall	0.50	0.51	0.51	0.51	0.016	0.965	0.149	0.622	<0.001	0.882

[a]Treatments were: (1) without SB supplement (CON); (2) SB supplement added to milk from 4 to 32 days (SB-4-32); (3) SB supplement added to milk from 61 to 74 days and added to starter from 75 to 88 days (SB-61-88) and (4) SB supplement added to milk (since days 4–74) and the starter (since days 75–88) for the total experiment (SB-4-88).
[b]Standard error of the mean.
[c]Days 4–74.
[d]Days 61–88.
[e]Days 75–88.
[f]g daily gain/g daily dry matter intake.

Feed efficiency did not show any difference among treatments in the current study. This achievement is in agreement with some studies (Kato *et al.*, 2011; Serbester *et al.*, 2014; Wanat *et al.*, 2015; Roh *et al.*, 2018; Ghaffari *et al.*, 2021) and in conflict with others (Hill *et al.*, 2007; Guilloteau *et al.*, 2010*b*; Nazari *et al.*, 2012; Davarmanesh *et al.*, 2015; Liu *et al.*, 2021). These discrepancies may be due to the type of used salt (calcium *v.* sodium) as well as how it was consumed; for instance, Davarmanesh *et al.* (2015) used calcium salt which was added to the MR until day 21 and then was included in the starter feed. Furthermore, it seems that the dosage plays a role in this regard; 3% of MR DM was evaluated in the study of Hill *et al.* (2007), while Kato *et al.* (2011) used an incremental dose of 3–7 g of SB.

More recently, Wu *et al.* (2022) reported that the addition of free SB to the starter feed was more effective than coated SB on performance and GIT development in the pre-weaning calves. The more efficient effects of free SB compared to the coated SB could be attributed to the different releasing sites in the GIT. Butyrate is metabolized by the ruminal epithelium primarily to beta-hydroxybutyric acid and is considered as an important energy source for the ruminal epithelial cells in the pre-weaning calves (Bergman, 1990; Wiese *et al.*, 2013). Furthermore, butyrate is considered to be the primary chemical promoter of rumen epithelium growth which reduces cell apoptosis (Mentschel *et al.*, 2001) and accelerates cell cycle progression in the ruminal epithelium cells (Sakata and Tamate, 1978; Malhi *et al.*, 2013). More

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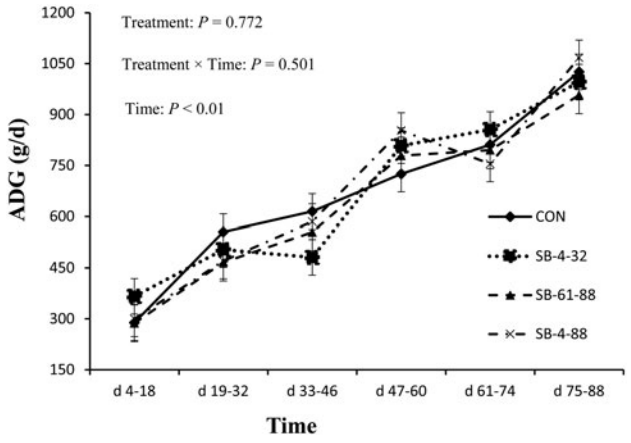

Fig. 2. ADG of calves supplemented with SB to WM. (♦) Control (CON) without micro-encapsulated SB supplementation; (■) with 4 g/day SB added to milk since days 4–32 (SB-4-32); (▲) with 4 g/day SB added to milk since days 61–74 and added to starter since days 75–88 (SB-61-88) and (×) with 4 g/day SB added to milk (since days 4–74) and the starter (since days 75–88) for the total experiment (SB-4-88).

research is warranted to determine if there is any difference between protected and un-protected supplemented SB to the WM. Furthermore, it is notable that the results of adding protected SB to WM should be interpreted with caution in the current study because butyric acid was previously included to the WM as unprotected. On the other hand, butyric acid was added to the MR or the starter feed in earlier studies.

Health criteria

Contrary to the initial hypothesis of the current study, butyrate did not affect diarrhoea or loose faeces during the first month of life but it was in agreement with our previous study (Mahjoubi *et al.*, 2020). On the other hand, lower faecal score and number of days with scours observed in our study are in line with previous studies which indicated that adding SB to MR and starter reduced diarrhoea and the number of days with scours (Hill *et al.*, 2007; Gorka *et al.*, 2009, 2011*b*; Guilloteau *et al.*, 2009*a*). Also, Sun *et al.* (2019) indicated that the occurrence

Table 4. Effects of SB supplementation to WM on structural growth indices of Holstein calves ($n = 12$ calves per treatment)

	Treatments[a]					*P*-value		
Item	CON	SB-4-32	SB-61-88	SB-4-88	S.E.M.[b]	Treat	Treat × time	CON *v.* SB-4-32, SB-61-88, SB-4-88
Withers height (cm)								
Day 4 (initial)	78.9	79.0	77.6	79.0	0.60	0.293	–	0.655
Day 32	85.2	86.3	84.7	85.1	0.54	0.210	–	0.851
Day 60	91.8	91.7	90.7	91.3	0.65	0.621	–	0.434
Day 74 (weaning)	94.4	94.6	93.1	94.1	0.52	0.219	–	0.466
Day 88 (final)	97.2	97.2	95.5	97.3	0.58	0.140	–	0.431
Hip height (cm)								
Day 4 (initial)	82.5	83.2	81.6	82.7	0.67	0.419	–	0.971
Day 32	88.6	89.4	88.1	88.5	0.52	0.431	–	0.939
Day 60	93.9	93.9	93.2	93.6	0.68	0.894	–	0.672
Day 74 (weaning)	95.8	96.0	94.9	95.5	0.58	0.596	–	0.660
Day 88 (final)	97.9	97.6	96.8	98.2	0.72	0.588	–	0.684
Heart girth (cm)								
Day 4 (initial)	77.7	78.4	78.0	77.8	0.53	0.747	–	0.526
Day 32	86.2	86.5	85.3	86.1	0.58	0.464	–	0.724
Day 60	96.2	95.2	93.9	95.5	0.78	0.243	–	0.154
Day 74 (weaning)	100	100	98	99	0.8	0.300	–	0.302
Day 88 (FINAL)	105.2	103.4	102.4	104.2	0.98	0.244	–	0.101
Body length (cm)								
Day 4 (initial)	46.1	46.4	45.3	46.1	0.53	0.510	–	0.805
Day 32	52.1	51.6	51.1	51.2	0.50	0.449	–	0.149
Day 60	57.8	56.6	57.1	57.2	0.62	0.566	–	0.228
Day 74 (weaning)	61.0	60.5	60.3	60.7	0.58	0.838	–	0.421
Day 88 (final)	63.9	62.9	62.6	63.6	0.53	0.303	–	0.153

[a]Treatments were: (1) without SB supplement (CON); (2) SB supplement added to milk from 4 to 32 days (SB-4-32); (3) SB supplement added to milk from 61 to 74 days and added to starter from 75 to 88 days (SB-61-88) and (4) SB supplement added to milk (since days 4–74) and the starter (since days 75–88) for the total experiment (SB-4-88).
[b]Standard error of the mean.

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Table 5. Mean values for health criteria and days experiencing a health criterion of Holstein calves supplemented with SB ($n = 12$ calves per treatment)

Item	Treatments[a]				S.E.M.[b]	*P*-value				Contrast
								Time		
	CON	SB-4-32	SB-61-88	SB-4-88		Treat	Treat × time	Linear	Quadratic	CON *v.* SB-4-32, SB-61-88, SB-4-88
Faecal score[c]										
Days 4–32	1.23	1.17	1.24	1.27	0.045	0.428	0.799	0.874	<0.001	0.960
Pre-weaning[d]	1.25[a]	1.17[b]	1.26[a]	1.18[b]	0.026	0.043	0.231	0.018	0.620	0.175
Transition period[e]	1.20	1.15	1.21	1.10	0.044	0.174	0.985	<0.001	0.016	0.340
Overall	1.23[a]	1.16[b]	1.24[a]	1.16[b]	0.023	0.034	0.365	0.091	0.074	0.142
Days with diarrhoea[f]										
Days 4–32	5.3	4.0	5.5	5.8	0.18	0.421	–	–	–	0.770
Pre-weaning	14.0[a]	9.5[b]	14.1[a]	10.2[b]	0.18	0.035	–	–	–	0.039
Transition period	3.3	2.7	4.2	1.6	0.24	0.187	–	–	–	0.570
Overall	15.0[a]	10.7[b]	15.0[a]	10.5[b]	0.19	0.025	–	–	–	0.066
General appearance[g]										
Days 4–32	1.07	1.04	1.05	1.07	0.027	0.877	0.342	0.593	0.237	0.652
Pre-weaning	1.04	1.03	1.04	1.03	0.010	0.844	0.167	0.276	0.029	0.867
Transition period	0.99	0.99	1.00	0.99	0.010	0.743	0.221	0.598	0.292	0.687
Overall	1.03	1.02	1.04	1.03	0.011	0.746	0.293	0.068	0.185	0.945
Days with elevated general appearance[h]										
Days 4–32	1.42	0.58	0.59	0.61	0.270	0.619	–	–	–	0.203
Pre-weaning	2.34	1.02	1.25	1.25	0.291	0.574	–	–	–	0.174
Transition period	0.00	0.00	0.09	0.00	0.210	0.121	–	–	–	0.480
Overall	2.34	1.02	1.32	1.21	0.295	0.593	–	–	–	0.189
Medical days[i]	0.62	0.72	0.90	0.41	0.334	0.931	–	–	–	0.953
Treatment bouts	0.28	0.21	0.32	0.19	0.192	0.958	–	–	–	0.848
Number of used drugs	0.54	0.60	0.86	0.52	0.310	0.963	–	–	–	0.830

[a]Treatments were: (1) without SB supplement (CON); (2) SB supplement added to milk from 4 to 32 days (SB-4-32); (3) SB supplement added to milk from 61 to 74 days and added to starter from 75 to 88 days (SB-61-88) and (4) SB supplement added to milk (since days 4–74) and the starter (since days 75–88) for the total experiment (SB-4-88).
[b]Standard error of the mean.
[c]1 = firm, 2 = soft, 3 = soft and running and 4 = watery.
[d]Days 4–74.
[e]Days 61–88.
[f]Days with faecal score ≥2; faecal score was square-root transformed and back-transformed values are presented in the table.
[g]1 = normal and alert; 2 = ears drooped; 3 = head and ears drooped, dull eyes, slightly lethargic; 4 = head and ears drooped, dull eyes, lethargic and 5 = severely lethargic.
[h]Days with general appearance score ≥2; general appearance was square-root transformed and back-transformed values are presented in the table.
[i]Treatment was carried out under on-farm protocol and according to the farm's veterinarian.
[a,b]Means within a row with different superscripts differ ($P < 0.05$).

of diarrhoea reduced when butyric acid was added to the acidified milk. In addition, Hill *et al.* (2007) found no effect of SB on medical days and the number of used antibiotic drugs (Gorka *et al.*, 2009, 2011*a*, 2011*b*). Less intestinal development, as a result of MR feeding instead of WM, makes newborn calves more prone to diarrhoea (Blattler *et al.*, 2001). Since WM was used in the current study, it seems that the amount of butyric acid in milk has partially led to the development of intestinal tissue thereby being able to handle diarrhoea. It has been shown that when butyric acid is added to MR, it improves colon function and can boost animal health (Guilloteau *et al.*, 2009*b*). Increased butyrate concentration in GIT by feeding molasses, it has been found that despite the increase in the concentration of butyric acid in the rumen, there was no effect on the faecal score (Oltramari *et al.*, 2016). Consistent with the present study, Wanat *et al.* (2015) also observed a linear effect with increasing butyric acid in the form of protected microcapsules on decreasing the faecal score. These results generally show that the effect of butyric acid addition to the starter depends on the level of supplementation and method of delivery which leads to obtaining contradictory results. In contrast with our expectation, although the addition of SB in the WM did not improve incidence of diarrhoea in the first month of life, it seems that the observed improvement in the faecal score

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Table 6. Effects of SB supplementation to WM on serum metabolites of Holstein calves ($n = 12$ calves per treatment)

Item	Treatments[a]				S.E.M.[b]	*P*-value				Contrast
								Time		
	CON	SB-4-32	SB-61-88	SB-4-88		Treat	Treat × time	Linear	Quadratic	CON *v.* SB-4-32, SB-61-88, SB-4-88
Pre-weaning										
Glucose (mg/dl)	111	113	111	113	2.3	0.889	0.288	<0.001	0.270	0.506
BHB (mmol/l)	0.14	0.16	0.14	0.16	0.011	0.508	0.581	<0.001	<0.001	0.386
TP (g/dl)	6.5	6.7	6.7	6.7	0.11	0.596	0.726	0.698	<0.001	0.229
Albumin (g/dl)	3.3	3.4	3.3	3.4	0.03	0.056	0.885	<0.001	0.608	0.032
Post-weaning										
Glucose (mg/dl)	95	95	84	93	3.9	0.152	–	–	–	0.345
BHB (mmol/l)	0.38	0.35	0.46	0.45	0.038	0.176	–	–	–	0.417
TP (g/dl)	6.7[b]	7.0[b]	7.6[a]	6.8[b]	0.17	<0.001	–	–	–	0.025
Albumin (g/dl)	3.4[b]	3.7[a]	3.5[ab]	3.7[a]	0.06	0.011	–	–	–	<0.001
Overall										
Glucose (mg/dl)	108	109	106	109	2.1	0.700	0.255	<0.001	0.039	0.819
BHB (mmol/l)	0.19	0.20	0.21	0.22	0.013	0.609	0.045	<0.001	<0.001	0.303
TP (g/dl)	6.6	6.7	6.9	6.7	0.10	0.143	0.201	0.018	<0.001	0.078
Albumin (g/dl)	3.3[b]	3.5[a]	3.4[ab]	3.5[a]	0.03	0.010	0.858	<0.001	0.725	<0.001

[a]Treatments were: (1) without SB supplement (CON); (2) SB supplement added to milk from 4 to 32 days (SB-4-32); (3) SB supplement added to milk from 61 to 74 days and added to starter from 75 to 88 days (SB-61-88) and (4) SB supplement added to milk (since days 4–74) and the starter (since days 75–88) for the total experiment (SB-4-88).
[b]Standard error of the mean.
[a,b]Means within a row with different superscripts differ ($P < 0.05$).

thorough the study is due to the long-term and carry-over effects of SB during pre-weaning.

Blood chemical items

Glucose is considered the preferred energy substrate in pre-ruminant calves (Donkin and Armentano, 1995). In agreement with previous studies (Ślusarczyk *et al.*, 2010; Roh *et al.*, 2018; McCurdy *et al.*, 2019; Mahjoubi *et al.*, 2020; Ghaffari *et al.*, 2021) we did not observe any effect of SB on glucose concentration. However, Kato *et al.* (2011) and Frieten *et al.* (2017) showed that butyric acid has potential to increase tissue sensitivity to insulin and decreases blood glucose concentration. Gorka *et al.* (2011*a*) showed that calves fed WM had more glucose in their blood than calves fed MR; also, the addition of butyric acid caused a significant increase in glucose concentration (Gorka *et al.*, 2011*a*, 2011*b*; Nazari *et al.*, 2012), which is contrary to the present study. Most likely, the reason for this variation is related to the length of the study or the amount of SB consumed. In the present study calves were weaned at day 74, while Gorka *et al.* (2011*a*, 2011*b*) slaughtered the experimental calves at 26 days of age.

The BHB concentration is an indicator of active rumen development in infant calves (Kristensen *et al.*, 2007). Mahjoubi *et al.* (2020) indicated an increase in BHB concentration when calves fed 4 or 8 g/day of SB were added to milk. This increase in BHB also indicates better ruminal function and development, so it affected the starter consumption of these groups. It was shown that BHB is produced by the rumen epithelium in well-fed ruminants (Pennington, 1952); accordingly, greater serum BHB indirectly indicates that SB leads to more extensive development in SB-fed calves. In agreement with the current results, previous studies (Ślusarczyk *et al.*, 2010; Davarmanesh *et al.*, 2015; Frieten *et al.*, 2017; Ghaffari *et al.*, 2021) reported no effect on BHB by adding butyric acid to milk substitute but not others (Nazari *et al.*, 2012; Roh *et al.*, 2018).

Post-weaning albumin and TP increased in SB-fed calves, which is in line with previous reports (Mahjoubi *et al.*, 2020). However, this observation is in contrast with prior studies in which SB feeding did not have impact on blood TP concentration (Sun *et al.*, 2019; Ghaffari *et al.*, 2021). Higher serum TP in calves fed SB also should be considered as a positive effect of SB supplementation, indicating greater accessibility of proteins for the developing organism. The response of albumin and TP to SB during the post-weaning period is probably because of the beneficial effect of butyric acid on intestine health (Gorka *et al.*, 2014) for the time of the transition period in which intestine permeability increases (Wood *et al.*, 2015; Adab *et al.*, 2020) and it may negatively interfere with the nutrient absorption.

Conclusion

At least in the first weeks of a calf's life, most of the butyrate supplemented in a protected form is expected to bypass the forestomach and abomasum and be delivered to the small intestine, where it exerts a local effect on the intestinal epithelium. However, supplementation of WM with SB did not have a noticeable effect on the feed intake and growth performance of dairy calves during the pre- and post-weaning periods in the current

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study. However, faecal score and the number of days with loose faeces decreased as SB was added to milk within early weeks of life (SB-4-32) and entire period of experiment (SB-4-88) in the pre-weaning period, and throughout the study. Under the condition of this study, although the faecal score and some blood metabolites were affected, but contrary to our hypothesis, supplementation with encapsulated SB in WM could not significantly affect calf performance, medical days and health status when it was fed during the first month of life. Given that the previous studies used the unprotected form of butyrate in WM, further research is needed to indicate clear difference between effects of addition of free or encapsulated SB into WM.

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Authors' contributions. All authors conceived and designed study. M. M. Eskandary conducted data gathering. M. Hossein Yazdi performed statistical analyses. M. Hossein Yazdi and E. Mahjoubi wrote the first draft of the manuscript. M. Kazemi-Bonchenari critically revised the paper. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. All experimental procedures were approved by the Ethics Committee on Animal Science at Arak University (IACUC no. IR2018011) and, in compliance with those norms, the animals did not suffer during the experimental procedures.

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