Butyrate supplementation in the liquid diet of dairy calves leads to a rapid recovery from diarrhea and reduces its occurrence and relapses in the preweaning period

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ABSTRACT

The present study aimed to evaluate the effect of continuous butyrate administration in dairy calves’ liquid diet considering diarrhea, metabolic profile, gastrointestinal development, and corporal growth. Immediately after birth, calves were randomly allocated into 2 groups of 62 calves (50 females and 12 males), with access to water and a solid feed ad libitum. The butyrate group (BG) received 4 g/d of sodium butyrate (Admix Easy, Adisseo) diluted in the whole milk, and the control group (CG) received whole milk with no supplementation. Sodium butyrate was administered from d 1 of life until the weaning at 90 d. Feces consistency was assessed daily for the first 30 d of life and characterized by scores from 0 to 4 (0 and 1 for normal, and 2, 3, and 4 for abnormal feces). Diarrhea was diagnosed when the animals had abnormal feces and fever. Morbidity, recurrence, mortality, and lethality data were recorded and compared between the groups. Average daily gain (ADG) and corporal growth (body weight, thoracic perimeter, height at the withers, and croup width) were evaluated weekly, from the first day to d 30, and later at 45, 60, and 90 d of life. Blood samples were taken weekly for up to 30 d to determine the circulating levels of total calcium, phosphorus, chloride, bicarbonate, glucose, β-hydroxybutyrate, and nonesterified fatty acids. The males were euthanized at 15 (n = 6 per group) and 30 d (n = 6 per group) for morphometric, histological, and gene expression analysis of the gastrointestinal tract. The results showed that the BG had a lower rate of morbidity (BG = 30% vs. CG = 50%) and recurrence (BG = 26.7% vs. CG = 60%) of diarrhea than the CG. In addition, the BG had abnormal feces for a shorter period (BG = 4.64 ± 0.47 d vs. CG = 8.6 ± 0.65 d). The ADG tended to be higher in BG than CG up to 30 and 60 d. Metabolic evaluations showed the lowest levels of glucose and highest levels of nonesterified fatty acids in BG. On d 30 of life, rumen papillae length, papilla area, duodenum villus length, and crypt depth were higher in BG than in CG. The duodenal gene expression at 30 d showed that animals with diarrhea episodes that did not receive butyrate had the highest levels of transcripts for the LCT and GLP2 genes. In addition, in different ways, both butyrate and neonatal diarrhea affected the gene expression of IGF1, SLC5A1, and AQP3. These results allow us to conclude that continuous supplementation with sodium butyrate improves gastrointestinal development, reduces the occurrence of diarrhea, and makes clinical conditions milder with faster recovery, favoring a higher ADG in the first 30 and 60 d of life. Based on these results, we conclude that sodium butyrate can be indicated for liquid diet supplementation to accelerate gastrointestinal tract development and prevent severe cases of neonatal diarrhea, tending to improve average daily gain until weaning.

Key words: gastrointestinal development, gene expression, feces, metabolism

INTRODUCTION

The rearing of dairy heifer calves is essential in the life production cycle of the dairy cow. In this process, the neonatal period is a critical moment in which nutrition and management practices are crucial to prevent diseases and ensure development and survival until the weaning (Volpato et al., 2017). After birth, the new
environment is loaded with potential pathogens, and the newborn calf is susceptible to diarrhea, which is the leading cause of morbidity and mortality in preweaning dairy calves worldwide (Rosa et al., 2018; Urie et al., 2018). The main consequences of diarrhea in dairy calves are growth retardation, increased susceptibility to other diseases, genetic losses (Cho and Yoon, 2014), impairment of reproductive performance, and lower lifetime milk production (Urie et al., 2018). In addition, the occurrence of diarrhea can delay the development and bacterial colonization of the gastrointestinal tract (GIT) in the preweaning period (Dias et al., 2018).

The development and maturation of the GIT occurs initially under the stimulation of a liquid diet (colostrum and milk) and later by solid foods (roughage and concentrate) (Guilloteau et al., 2009). The butyrate has been pointed out as a key part of these processes (Dias et al., 2018). Butyrate is a short-chain fatty acid naturally produced in the rumen and hindgut in ruminants (Górka et al., 2018). These sections of the GIT are colonized by a great diversity of bacteria, fungi, archaea, and viruses known as the microbiota. The microbiota’s diversity is influenced by internal and external factors that modulate its composition and function (de Moraes et al., 2014). These colonizing microorganisms can play a role in hormone production (Wijdeveld et al., 2020), affect feeding, modulate digestive and absorptive processes, metabolism, and immune response, with repercussions on energy, homeostasis, health, and reproduction of hosts (Wijdeveld et al., 2020).

After providing a butyrate supplementation to newborn calves, Hill et al. (2007) observed an increase in size and number of ruminal papillae, as well as an increase in the development of intestinal epithelium villi and crypts of the calves. Still, according to Hill et al. (2007), ruminal and intestinal changes in calves produced by butyrate supplementation favored food digestion, improved the intestinal absorptive area, fecal consistency, and the growth of the animals. In addition, the supply of sodium butyrate in the liquid diet of cattle in the initial phases of the development could be linked to other effects, including the increase in pancreatic juice production, antisecretory and antiinflammatory effects in the intestine, changes on the somatotropic axis, and energy metabolism (Guilloteau et al., 2009; Canani et al., 2011; Górka et al., 2018).

Although the influence of butyrate on the GIT development has already been reported by several groups (Guilloteau et al., 2009; Górka et al., 2011a), there are few studies investigating a possible preventive effect of dietary butyrate supplementation on diarrhea in dairy calves (Hill et al., 2007; Górka et al., 2011a). Also, data of the effects of neonatal diarrhea and butyrate supplementation on the regulation of genes related to the lactose digestion, cell membrane transporters, and cell proliferation and repair in the small intestine are lacking.

We hypothesized that, in addition to promoting GIT development, butyrate may act to prevent diarrheal conditions, contributing to the growth of animals during the preweaning period.

The present study aimed to evaluate the effect of continuous administration of butyrate in the liquid diet of dairy calves on the clinical manifestations of diarrhea, metabolic profile, GIT development, and corporal growth.

**MATERIALS AND METHODS**

**Experimental Design**

All procedures in this study were approved by the Animal Ethics and Experimentation Committee of the Federal University of Pelotas (no. CEUA 9466–2020). The study was conducted on a commercial farm with an intensive milk production system in Rio Grande, RS, Brazil (32° 16’ S, 52° 32’ E). Holstein dairy calves were used for the study (100 females and 24 males, n = 124). Soon after birth, identification was performed with earrings, and the animals were housed in individual pens containing rice husk bedding. Four liters of colostrum in one portion with a quality equal to or greater than 25 degrees Brix were provided for the calves within the first 12 h after birth. Also, 10% iodine was applied to the navel twice a day for the first 3 d. After that, the animals were transferred to suspended pens made of wood and kept in pairs until 90 d, fed daily with 6 L (~15% BW) of milk (3 L in the morning and 3 L in the afternoon) and with access to water and solid feed ad libitum. The farm diet was similar to that used in previous studies (Slanzon et al., 2019). The milk supplied had 12.5% average DM. The DM content (g/kg), the analyzed chemical composition (g/kg DM), and the solid feed levels provided during the experiment are shown in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.23706690.v1).

The 124 animals were homogeneously randomized into 2 groups by birth order and weight. The butyrate group (BG; 50 females and 12 males; n = 62; birth weight = 38.9 ± 2.1 kg) received daily a commercial product containing 90% of sodium butyrate (Admix Easy, Adisseo) added to the milk, throughout the preweaning period, following the manufacturer’s recommendation (4 g/d, 0.5% of milk DM). The control group (CG; 50 females and 12 males, n = 62, birth weight = 38.8 ± 2 kg) consisted of animals that received milk without any additive.

The failure of passive immunity transfer was used as an exclusion criterion for animals in this study.
this, between 24 and 48 h of life, blood was collected by jugular venipuncture using a Vacutainer System (BD Diagnostics, São Paulo, Brazil) and tubes containing EDTA. The whole blood was centrifuged to obtain and analyze the total plasmatic protein concentration, and values of at least 5.5 g/dL of PPT (corresponding to 1 g/dL serum IgG) were used as a cut-off point (Tyler et al., 1999).

Feces Score Evaluation

Feces consistency was determined daily up to 30 d of age in females and males (n = 124), using the following score: 0 (normal), 1 (loose), 2 (watery), 3 (profuse diarrhea with liquefied feces), and 4 (profuse diarrhea with liquefied and bloody feces). Based on the feces score, animals with values equal to or above 2 (2 to 4) were considered to have diarrheal wastes (McGuirk, 2008). Also, other possible clinical changes, such as fever or dehydration (Radostits et al., 2006), were considered for diagnosing diarrhea. The initial treatment of diarrhea was carried out with an oral solution of activated charcoal. In animals that presented fever higher than 40.5°C and apathy, systemic treatment was performed with flunixin meglumine and sulfa with trimethoprim according to the package leaflet instructions. The day diarrhea ended was considered the day the animal again presented feces scores of 0 or 1.

Disease Monitoring During the Preweaning Period

The females (n = 100) were monitored until 90 d of age and the males (n = 24) until euthanasia for the occurrence of diarrhea, determining morbidity (number of animals that became ill/total number of animals in the experiment), mortality (number of animals that died/total number of animals), lethality (number of animals that died from diarrhea/number of animals that had diarrhea), and recurrence (number of animals that became sick twice or more during the period considered). Diarrhea in males was also monitored until 15 and 30 d of age.

The occurrence of respiratory diseases, as well as mortality from this disease, was also determined. Furthermore, other neonatal diseases were evaluated, generating the incidence of each condition and the overall mortality (total number of animals that died/total number of animals) (Radostits et al., 2006).

Zootechnical Evaluations

Assessments of weight, thoracic perimeter, height at the withers, and rump width of the females were performed at birth and weekly until 30 d of age and later at 45, 60, and 90 d of age. The ADG of animals that completed 90 d of age (100 animals, minus deaths) was determined through BW.

Collection of Tissue Fragments from the Gastrointestinal Tract

Aiming to evaluate possible changes in GIT morphology under the influence of butyrate and to determine the period in which they would occur (in the first 15 d or between 15 to 30 d of life), tissues were collected for histological analysis. Thus, at 15 and 30 d of age, 24 male calves were euthanized to evaluate the development of the gastric and intestinal compartments, totaling 12 animals at d 15 (n = 6 BG and n = 6 CG) and 12 animals (n = 6 BG and n = 6 CG) at d 30 of life. The number of animals for carrying out the histological analyses was determined based on previous studies (Liu et al., 2018). The performance of euthanasia followed the recommendations of the Federal Council of Veterinary Medicine (FCVM) expressed in Resolution No. 1000 of May 11, 2012. After confirmation of death, rumen-reticulum, omasum, and abomasum were individualized, emptied, washed repeatedly with water, dried, and weighed. Tissues samples of 1 cm² were collected in duplicate for histological and molecular analysis. The locations of the tissue collection were always the same for all the animals, including ventral sac of the rumen, and mid-ventral section of the abomasum, duodenum (about 10 cm distal to the pyloric sphincter), jejunum (10 cm distal to the duodenal-jejunal ligament), ileum (10 cm proximal to the ileocecal junction), and colon (medial portion) (Liu et al., 2018).

Histological Analyses

The samples were sequentially dehydrated in 80%, 90%, and absolute alcohol, cleared in xylene, and incorporated into paraffin blocks during processing. Thick sections (10 sections of each sample) were taken from each piece with a microtome (RM 2245, Leica Biosystems Nussloch GmbH, Germany). These were distended on microscopy slides and stained with hematoxylin and eosin to be mounted (Entellan, Merck, Germany) with coverslips. Images from slides were captured by a camera (Moticam 5, 5.0 MP, USB, Motic, China) attached to a microscope (Eclipse E200, Nikon, Japan). The mor-
phometry of the rumen papillae (length, width, surface, and density) and intestinal villi (height, depth of the crypt, and villus-crypt ratio) were determined in 30 papillae (rumen) and 30 villi with corresponding crypts (intestine) using the image analysis of the software (Motic Images Plus 2.0, Motic, China). The length of the ruminal papilla was measured from the apex to the base, whereas the width was in the middle of the papilla (Hofmann and Schnorr, 1982). Density was evaluated with a magnifying glass (SM45TR, Physix) attached to the video camera (Motimac 5, 5.0 MP, USB, Motic) and determined further using the Image J (Image J 1.44 software, National Institutes of Health, Bethesda, MD). The surface of the papillae per centimeter² was determined as length × width × 2. In the intestine, the villus height was measured from the villus tip to the villus-crypt interface, whereas the crypt depth was calculated from the villus-crypt to the lamina propria opening at the base of the villi (Schäff et al., 2018).

**Gene Expression Analysis**

Expression analysis of different genes was performed to assess whether episodes of neonatal diarrhea or butyrate supplementation (or both) could influence intestinal integrity and differentiation through genes that regulate functions such as cell proliferation, transmembrane transport in enterocytes, and production of the enzyme lactase.

As the main morphometric changes observed in response to butyrate supplementation through histological analysis occurred in the duodenal portion and at 30 d of life, the samples collected from this intestinal section and at this time were chosen for gene expression analyses. Thus, duodenal tissues from animals euthanized at 30 d of age in the CG (n = 6) and BG (n = 6), diagnosed with (n = 3) and without (n = 3) diarrhea during the neonatal period, had transcripts levels for the genes glucagon-like peptide 2 (GLP2), lactase (LCT), insulin-like growth factor 1 (IGF1), solute carrier family 5 member 1 (SLC5A1), and aquaporin 3 (AQP3) analyzed (Supplemental Table S2; https://doi.org/10.6084/m9.figshare.23706690.v1). The causes were not clarified, and the diarrheic episodes occurred on d 17, 18, and 28 of life in the CG and d 4, 11, and 13 of life in BG. The main rectal temperature registered for the diarrheic episodes was 40.8 in CG and 41.1 in the BG.

The genes AQP3 and SLC5A1 are cell membrane transporters of the intestine. The AQP3 encodes the aquaporin 3, an integral membrane protein expressed in the epithelium along the digestive tract that serves as a channel in the transfer of water in the cell membrane. The SLC5A1 gene encodes for the sodium/glucone cotransporter 1 protein (SGLT1), which is sodium dependent and transports monosaccharides (glucose/galactose) from the intestinal lumen across the cell membrane in a competitive process. Its intrinsic activity increases when there is elevated luminal glucose concentration. The LCT gene provides instructions for making lactase, the enzyme produced by epithelial cells of intestinal microvilli that helps to digest lactose. The IGF1 has a role in cell proliferation. The GLP-2 gene promotes intestinal growth, tissue development, and repair.

The RNA extraction was performed using the PureLink kit (Invitrogen, Carlsbad, CA), with minor adaptions to the manufacturer’s instructions (30 mg of tissue, 600 µL of lysis buffer, and final elution with 30 µL in DEPC water). Total RNA quantification was performed using a NanoVue spectrophotometer (GE Healthcare, Chicago, IL). Through the absorbance ratio of 260/280 nm, a purity index of 2.0 to 2.04 was observed in all samples. Complementary DNA synthesis was conducted using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA) with an input of 1 µg of total RNA, to a final volume of 20 µL, following the manufacturer’s instructions.

Real-time PCR was performed in the StepOne system (Applied Biosystems), using 1 µL of pure cDNA, qPCR Mix, and CXR reference probe (GoTaq qPCR MasterMix, Promega, Madison, WI), mixed to specific primer pairs (Exxtend, Paulínia, São Paulo, Brazil) with 0.25 µM concentration. The final volume of the qPCR reaction was 12.5 µL, and all reactions were performed in duplicate. Thermocycling conditions followed the manufacturer’s instructions.

The differences in the gene expression levels of duodenal samples from groups BG, CG, diarrheal BG, and nondiarrheal CG at 30 d of age (n = 4 animals per group) were determined using the 2−ΔΔCT method (Livak and Schmittgen, 2001). The relative expression of the target genes was normalized with the RN18S1 gene (Supplemental Table S2).

**Blood Collection and Metabolic Assessments**

Blood collection was performed in females through jugular venipuncture on d 1, 8, 15, 22, and 29 from 20 females in each group (Reis et al., 2022). Tubes without anticoagulants were used to assess serum levels of total calcium, phosphorus, chloride, bicarbonate, BHB, and nonesterified fatty acids (NEFA), and tubes with potassium fluoride for lactate and glucose assessment. Immediately after collection, blood samples were centrifuged at 2,183 × g and 15°C for 10 min to obtain serum and plasma. Subsequently, serum and plasma were placed in 1.5-mL Eppendorf-type microtubes (in
duplicate) and frozen. Later, the collected serum and plasma samples were evaluated using Plenno enzyme kits (Labtest Diagnóstica, MG, Brazil) in a Labmax Plenno automatic biochemical analyzer (Labtest Diagnóstica).

**Statistical Analysis**

The normality of the data ($F > 0.90$) was evaluated with the Shapiro-Wilk test. Continuous variables with multiple collections such as zootecchnical evaluations, gastrointestinal morphometrics, and metabolic parameters were evaluated using the GLIMMIX model, considering the animal, group, collection time, and interactions. Continuous variables with a single collection, such as the ADG, were analyzed using the $t$-test. Categorical variables were evaluated using the chi-squared test. All the analyses above were performed in the SAS statistical program (JMP Pro 14.0, SAS Institute Inc., Cary, NC). The results of differential gene expression, considering the group and the occurrence of neonatal diarrhea, were obtained by 2-way ANOVA using the GraphPad Prism v.8 software (San Diego, CA). Differences between groups were considered significant when $P < 0.05$ and as a trend when $P > 0.05$ and $< 0.1$.

### RESULTS

**Occurrence of Disease and Mortality**

When comparing the averages of diarrhea morbidity, considering the total preweaning period (90 d), a difference was observed between the control and butyrate groups ($CG = 50\%$ vs. $BG = 30\%$, $P = 0.04$). There was also a lower recurrence of cases in BG compared with control ($CG = 60\%$ vs. $BG = 26.67\%$, $P = 0.04$). The comparison results between morbidity, mortality, lethality, and disease recurrence rates can be seen in Table 1.

### Feces Score

The feces score was used to characterize the time that the calves of each group remained diarrheic (score $\geq 2$) during the first 30 d of life. As a result, the BG showed a reduction in days with diarrhea ($P < 0.001$) compared with the CG (Figure 1).

### Zootecchnical Evaluation

In the zootecchnical evaluations, there was a tendency ($P = 0.07$) toward a higher ADG when considering the neonatal period, up to 28 d, and a trend ($P = 0.09$) from 0 to 60 d. In the other periods, the ADG of the BG was equivalent to that of the CG ($P > 0.1$; Table 2). The other zootecchnical parameters of croup width, thoracic perimeter, and height at the withers were not influenced by butyrate supplementation in the calves’ diet ($P > 0.1$; Table 3).

The achievement of twice the birth weight at 60 d and at 90 d was evaluated. Twice the birth weight is the index used as a parameter for weaning. At 60 d of age, there was a tendency ($P = 0.06$) for a higher percentage of animals in the BG to reach twice their birth weight than in the CG (25% vs. 10.64%). The farm weaned most of the animals at 90 d of age, and at this moment, 93.75% of the animals in the BG and 91.3% of the CG presented double or more than birth weight, with no difference between groups ($P > 0.1$).

### Morphometric Assessments of the Gastrointestinal Tract

The histological evaluations aimed to measure the development of the GIT. Size, density per centimeter$^2$, width, area of the ruminal papillae, villi size, and depth of the crypts in the duodenum, jejunum, and ileum were evaluated at d 15 and 30 of age. The results with
P-values for the difference between groups, age, and interaction are shown in Figures 2 and 3.

In the rumen, a greater \( P < 0.01 \) papilla length was observed in BG compared with CG at 30 d of life (Figure 2A), which reflected in the largest \( P < 0.01 \) area of papilla (Figure 2C). At this time, the density of papillae per centimeter\(^2\) was also higher \( P < 0.01 \) in BG compared with CG. As for the width of the papillae, there were no differences between the groups \( P > 0.05 \), only between the ages (Figure 2B).

In the small intestine, a difference was observed between the groups only in the duodenum section, where the villus length in BG was greater \( P < 0.01 \) than in the CG, at 30 d of life (Figure 3A). The same was observed for the crypt depth (Figure 3B).

Regarding the total weight of the gastric compartments (reticulum, rumen, omasum, and abomasum), there were no differences between the groups.

**Gene Expression Analysis**

Neonatal diarrhea as well as the butyrate supplementation and their possible influences in duodenal epithelium of calves were evaluated through the expression of genes related to the cell membrane transporters, cell proliferation and repair, and lactose digestion. The relative expression of \( IGF1 \), \( LCT \), \( GLP2 \), \( SLC5A1 \), and \( AQP3 \) genes in the duodenum are shown in Figure 4.

Butyrate supplementation in the liquid diet induced effects on gene expression in calves that did not have diarrhea during the neonatal period. Animals without diarrhea and that were not supplemented had greater \( P < 0.05 \) expression of \( IGF1 \). On the other hand, animals supplemented with sodium butyrate, with diarrhea, had higher \( P < 0.05 \) expression levels for \( IGF1 \) and \( SLC5A1 \) genes.

The occurrence of neonatal diarrhea also influenced the expression of genes in nonsupplemented animals at 30 d of life. In animals that received milk without butyrate and that did not have diarrhea, lower \( P < 0.05 \) levels of expression of \( LCT \), \( GLP2 \), and \( SLC5A1 \) were found than in those with neonatal diarrhea. Also, in these animals, there was greater \( P < 0.05 \) expression of the \( AQP3 \) genes compared with animals with diarrhea.

The occurrence of neonatal diarrhea also influenced gene expression in animals supplemented with butyrate. In this case, only the \( SLC5A1 \) genes differed \( P < 0.05 \) between animals with and without diarrhea, both

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**Figure 1.** Average days with abnormal feces in dairy heifers supplemented or not with butyrate during the first 30 d of life (n = 100). Feces were considered abnormal when scores ≥2 were identified on the following scale: 0 (normal feces), 1 (loose feces), 2 (watery feces), 3 (profuse diarrhea with liquefied feces), and 4 (profuse diarrhea with liquefied feces and bloody content). Error bars indicate SEM.

**Table 2.** Mean daily weight gain (ADG; kg) of animals supplemented or not supplemented with butyrate during lactation, broken down by period (n = 95)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Butyrate</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>ADG up to 8 d</td>
<td>0.340 ± 0.036</td>
<td>0.401 ± 0.028</td>
<td>0.17</td>
</tr>
<tr>
<td>ADG from 8 to 15 d</td>
<td>0.364 ± 0.040</td>
<td>0.417 ± 0.035</td>
<td>0.33</td>
</tr>
<tr>
<td>ADG from 15 to 22 d</td>
<td>0.477 ± 0.040</td>
<td>0.548 ± 0.039</td>
<td>0.22</td>
</tr>
<tr>
<td>ADG from 22 to 29 d</td>
<td>0.492 ± 0.043</td>
<td>0.524 ± 0.050</td>
<td>0.63</td>
</tr>
<tr>
<td>ADG from 29 to 45 d</td>
<td>0.533 ± 0.043</td>
<td>0.551 ± 0.030</td>
<td>0.66</td>
</tr>
<tr>
<td>ADG from 45 to 60 d</td>
<td>0.740 ± 0.030</td>
<td>0.761 ± 0.029</td>
<td>0.67</td>
</tr>
<tr>
<td>ADG from 60 to 90 d</td>
<td>0.835 ± 0.037</td>
<td>0.798 ± 0.035</td>
<td>0.50</td>
</tr>
<tr>
<td>ADG from 0 to 29 d</td>
<td>0.394 ± 0.018</td>
<td>0.441 ± 0.018</td>
<td>0.07</td>
</tr>
<tr>
<td>ADG from 0 to 60 d</td>
<td>0.516 ± 0.016</td>
<td>0.555 ± 0.016</td>
<td>0.09</td>
</tr>
<tr>
<td>ADG from 29 to 90 d</td>
<td>0.743 ± 0.024</td>
<td>0.727 ± 0.024</td>
<td>0.64</td>
</tr>
<tr>
<td>ADG during preweaning</td>
<td>0.627 ± 0.019</td>
<td>0.632 ± 0.017</td>
<td>0.84</td>
</tr>
</tbody>
</table>

\( ^{A,B} \)Different letters within a row indicate a trend \( P < 0.1 \).
Compared with nonsupplemented control without diarrhea, the calves with diarrhea and supplemented with sodium butyrate had equivalent (P > 0.05) levels of expression for all genes studied, except for AQP3.

**Metabolic Assessments**

In the metabolic evaluations, a higher concentration of glucose (P < 0.01) was observed in the CG in relation to BG when all analyzed periods were considered, whereas the serum concentration of NEFA was higher (P < 0.01) in the BG (Figure 5). The other parameters evaluated were not different between the groups. Figure 6 shows the results of glucose and NEFA, also considering the occurrence or not of diarrhea during the 30 first days.

**DISCUSSION**

To the best of our knowledge, the present study is the first to demonstrate in a single experiment, the effects of sodium butyrate on body growth, occurrence of

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**Table 3. Zootchnical parameters of calves fed milk supplemented or not with butyrate for 90 d of life (n = 95)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Butyrate</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average SEM</td>
<td>Average SEM</td>
<td>Group Day Group × day</td>
</tr>
<tr>
<td>Thoracic perimeter (cm)</td>
<td>85.09 0.39</td>
<td>85.63 0.39</td>
<td>0.33 &lt;0.01 0.99</td>
</tr>
<tr>
<td>Height at withers (cm)</td>
<td>82.67 0.35</td>
<td>82.67 0.34</td>
<td>0.99 &lt;0.01 0.59</td>
</tr>
<tr>
<td>Croup width (cm)</td>
<td>21.80 0.13</td>
<td>22.04 0.13</td>
<td>0.18 &lt;0.01 0.73</td>
</tr>
</tbody>
</table>

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**Figure 2.** Evaluation of the ruminal development of calves fed milk supplemented with butyrate (butyrate; n = 12) or not (control; n = 12). (A) ruminal papilla length, (B) ruminal papilla width, (C) ruminal papilla area, (D) ruminal papilla width density. Different letters indicate a significant difference (P < 0.05). Error bars indicate SEM.
Figure 3. Evaluation of the intestinal development of calves fed milk supplemented with butyrate (butyrate; n = 12) or not (control; n = 12). (A) duodenal villus length, (B) duodenal crypt depth, (C) villus/duodenal crypt ratio (V/C), (D) jejunal villus length, (E) jejunal crypt depth, (F) villus/jejunal crypt ratio (V/C), (G) ileum villus length, (H) ileum crypt depth, (I) villus/ileum crypt ratio (V/C). Different letters indicate a significant difference (P < 0.05). Error bars indicate SEM.
Figure 4. Relative expression of *IGF1*, *LCT*, *GLP-2*, *SLC5A1*, and *AQP3* transcripts in the duodenum of calves at 30 d of age. Groups of animals fed with milk (control; $n = 6$) or milk supplemented with butyrate (butyrate; $n = 6$) were diagnosed with (with diarrhea; $n = 3$ in each group) and without (without diarrhea; $n = 3$ in each group) diarrhea during the neonatal period. (A) *IGF1*, (B) *GLP2*, (C) *LCT*, (D) *SLC5A1*, (E) *AQP3*. Different letters indicate a significant difference ($P < 0.05$). Error bars indicate SEM.
Figure 5. Evaluation of the serum metabolic profile (glucose, nonesterified fatty acids [NEFA], lactate, BHB, calcium, phosphorus, chloride, and bicarbonate) of calves fed with milk supplemented with butyrate (butyrate; n = 20) or not (control; n = 20). (A) glucose, (B) NEFA, (C) lactate, (D) chloride, (E) phosphorus, (F) calcium, (G) bicarbonate, (H) BHB. Error bars indicate SEM.
diarrhea, gastrointestinal development, duodenal gene expression and serum metabolic profile of dairy calves. In Brazil, the morbidity and recurrence of diarrhea in dairy herds, in general, remain still high (Weiller et al., 2021; Reis et al., 2022). According to the cited authors and the review by Virgíno Júnior and Bittar (2021), the main causes of diarrhea are related to infectious agents, especially viral and food causes.

In our study, sodium butyrate supplementation in the liquid diet reduced morbidity and diarrhea recurrences. This can be attributed to a possible antibacterial effect provided by sodium butyrate. According to Cherrington et al. (1991), butyrate changes electrochemical gradients and reduces intestinal pH, affecting the colonization of pathogenic gram-negative bacteria, the leading cause of diarrhea (Drackley, 2008). Also, a shorter period with abnormal feces was observed in calves supplemented with sodium butyrate, from 8.26 to 4.64 d, similar to Hill et al. (2007), who observed a reduction from 10.2 to 9 d with abnormal feces in the first 28 d of life in Holstein calves supplemented with butyrate. The explanation may be related to a possible reduction in the infective pressure of pathogenic bacteria at the gastrointestinal level in response to supplementation (Guilloteau et al., 2010). Another reason could be related to the greater development of the rumen and duodenum observed in the histological evaluation of BG. More efficient absorption of nutrients and consequently better metabolic status allow for earlier recovery from diarrhea (Guilloteau et al., 2010).

Regarding health, the level of feeding can also be a determining factor. Todd et al. (2017), shows that calves fed with milk ad libitum are healthier in the preweaning compared with animals that receive a predetermined amount of milk. However, Gerbert et al. (2018) observed no difference between ad libitum and restrictive feeding in relation to the health of calves. In our study, the animals received 6 L of whole milk daily in both groups, demonstrating the beneficial effects of butyrate in reducing diarrhea in this feeding system.

The effects of butyrate in the gut are confirmed by many studies, but even with Górka et al. (2018) pointing in their review that supplemented butyrate in both milk and concentrate has beneficial effects on rumen development, until now, it was not clear whether butyrate given with milk could affect rumen development. Our study showed greater length of the papillae, and greater area and density of papillae per centimeter² in feeding calves with whole milk containing sodium butyrate. In the study of Koch et al. (2019) the epithelial growth was not influenced by oral butyrate administration with milk replacer. However, the study of Górka et al. (2011b) reported a shorter papilla length in the cranial dorsal sac in milk replacer–fed calves than in calves fed whole milk. Whole milk is a complete and complex composition with several hormones, antibodies, glycans, glycoconjugates, antimicrobials, and an excellent substrate source for gut microbiota growth (Coelho et al., 2022) that together with butyrate, which increases secretion of peptides and hormones in the GIT (Guilloteau et al., 2010; Górka et al., 2018), could increase the development in BG calves from our study.

**Figure 6.** Evaluation of the serum metabolic profile (glucose and nonesterified fatty acids [NEFA]) in dairy heifers during the first 30 d of life. Groups of animals fed with milk (control; n = 20) or milk supplemented with butyrate (butyrate; n = 20) were diagnosed with and without diarrhea during the neonatal period. (A) Glucose, (B) NEFA. Different letters indicate a significant difference ($P < 0.05$). Error bars indicate SEM.
The exact role of milk containing butyrate in calf rumen development during early life is unclear and needs to be established. In this respect, microRNAs can be an interesting line of investigation. Bta-miR-493 has been pointed as the most significant different expressed miRNA between the preweaning and postweaning period (Do et al., 2019). Also, its expression was abundant in rumen and small intestinal (mid-jejunum and ileum) tissue samples at 30 min after birth, 7 d, 21 d, and 42 d of calf’s life (Liang et al., 2014). The function of bta-miR-493 in bovine is unclear but it targets genes involved primarily in the proliferation of connective tissue cells and muscle cells, suggesting a role in regulating rapid tissue development during the early life of calves (Liang et al., 2014). The effect of butyrate milk feed on the modulation of microRNAs, including bta-miR-493, may be the key to understand the mechanisms behind the ruminal development found in our study in butyrate-fed calves, and we have plans to study this in the near future.

The greater intestinal villi length and crypt depth increases the absorption surface area; consequently, there is greater use of the ingested nutrients with butyrate supplementation in bovine diets during preweaning (Górka et al., 2018). In our study, at 30 d of life, villi development was higher in the duodenum due to butyrate supplementation. Gerbert et al. (2017) also found an effect of butyrate supplementation on the growth of intestinal villi. These effects can also explain the increase in consumption observed by McCurdy et al. (2019). Still, unfortunately, it was not possible to evaluate consumption of the solid feed in our study.

Regarding gene expression, the IGF1 gene, which regulates growth, was found to be downregulated in calves supplemented with butyrate compared with the CG. Greater development of intestinal villi and lower expression of IGF1 in calves supplemented with butyrate was also reported by Koch et al. (2019). These results suggest that villi proliferation and development at 30 d of life can be not only mediated by the IGF1 gene, or that the IGF1 gene expression levels decreased before the time of collection (30th day of life).

The lactase gene (LCT) related to the consumption of a diet rich in lactose encodes the lactase enzyme, which is part of the intestinal secretion of young mammals and essential for the digestion of milk (Le Huerou et al., 1992). Lactase activity is high at birth and then declines, especially after weaning once levels of mRNA and lactase activity are influenced by nutrition (Ontsouka et al., 2004). The highest levels of transcripts for the LCT gene in our study were observed in the CG with diarrhea. This result contrasts with the downregulation of the LCT transcripts in BG with diarrhea that despite having suffered from this condition, had similar LCT expression to those of the CG without diarrhea in the first 30 d of life. Considering the importance of lactase in milk digestion (Le Huerou et al., 1992), the reduced expression of LCT found at 30 d of age in calves suggests a lower demand for intestinal lactase, proportional to the development of the GIT and the consumption of solid foods (Nicola et al., 2022). Therefore, in the calves with diarrhea, the LCT expression level was lower in BG than in CG, which suggest less dependence on liquid feed and probably greater consumption of solid feed in this group in comparison to the CG. Unfortunately, as stated above, evaluating DMI was not possible because the animals were housed in pairs, and we could not evaluate individual feed consumption. This is a limitation of our study. Still, the results suggest a predominance of solid feed consumption over a liquid diet, both in the CG that did not have diarrhea and in the BG that had neonatal diarrhea.

The GLP2 gene encoding the hormone GLP-2 that uses both IGF-1 and epidermal growth factor (EGF) as intermediary factors to promote intestinal growth, tissue development, and repair (Burrin et al., 2005). When administered exogenously in healthy animals of monogastric species, GLP-2 promotes intestinal epithelium growth by increasing villi and crypts, reducing epithelial cell apoptosis, and improving gut blood flow, nutrient absorption, and epithelial barrier function. Also, GLP-2 stimulates intestinal blood flow and produces antiinflammatory effects in addition to this cell proliferation action (Dubé and Brubaker, 2007).

Both BG (with or without diarrhea) showed expression values for the LCT and GLP2 genes like those observed in the CG without diarrhea. Considering the BG animals had greater intestinal development at 30 d, is possible to assume that they are more prone to consume the solid feed (Le Huerou et al., 1992). In this sense, further studies could evaluate the potential application of these genes as molecular biomarkers of gut maturity and investigate these levels in pre- and postweaning. Furthermore, recent studies have suggested that GLP-2 has a protective effect on intestinal permeability and the subsequent infiltration of endotoxins (Kvidera et al., 2017), thus explaining the higher expression of GLP2 gene in the CG with diarrhea. Also, the high GLP2 expression is due to a demand for local tissue repair caused by the inflammatory process and increased intestinal permeability in the animals that had diarrhea. In the BG with diarrhea, the reduced expression of GLP2 may suggest that the demand has been lower than CG with diarrhea.

Since our study found that the BG animals remained with diarrheal feces for fewer days, it is possible that the increase in intestinal permeability and the local
inflammatory stimulus in this group were minimal. The downregulation of GLP2 on d 30 of life suggests that tissue repair after diarrhea took place in a shorter period in BG, leading this group of animals to have a status in terms of gene expression equivalent to that of animals that crossed the entire neonatal period in the absence of diarrhea (Nicola et al., 2022).

The AQP3 gene is a stimulator of forming aquaporins, channels responsible for transporting water through the intestinal wall (Rojek et al., 2008). In our study, the expression of AQP3 gene was modulated by diarrhea and not by butyrate. This reduction was previously found in rats with colitis by Zhao et al. (2014) and in calves with mild inflammatory diarrhea by Rosa et al. (2018), indicating a reduced water exchange between the intestinal lumen and the epithelium in this case.

The SLC5A1 gene is involved in transporting glucose from the intestinal lumen to the body (Wright, 2013). Our findings indicate that its upregulation was stimulated by a compensatory effect on the occurrence of diarrhea, unlike other studies (Rosa et al., 2018), which found a downregulation on SLC5A1 gene expression in animals with diarrhea. This upregulation of SLC5A1 in our study may have occurred because the collections were not carried out on the day of diarrhea. Still, at 30 d of life, when the conditions were no longer in the clinical course, the gene expression was higher due to a possible increase in energy demand to restore intestinal tissues damaged by the disease (Kvidera et al., 2017).

Overall, these data suggest that multiple adaptive changes occur in the mucosa after diarrhea.

Regarding glucose, our results showed physiological levels of glycemia in all the groups, but glucose concentration was lower in the BG without diarrhea (Figure 6). In the study carried out by Hatew et al. (2019), a lower serum glucose concentration in BG was also observed. The greater expression of the SLC5A1 gene in the duodenal tissue observed in the animals BG and CG with diarrhea may give an explanation to this, once this upregulation is a strong indication that there was a compensatory increase in the carbohydrates made up of carbon, hydrogen, and oxygen (CHO) uptake in these groups after diarrhea. This fact possibly contributed to a higher glucose availability in the bloodstream of these animals.

The SGLT1 protein encoded by the SLC5A1 gene is highly dynamic, with its activity modulated by multiple mechanisms to ensure maximal uptake of carbohydrates by enterocytes. In both groups (control and butyrate), we observed in animals with diarrhea that intestinal mucosal SLC5A1 mRNA was increased, and with this, physiological glucose absorption probably was enhanced.

In the short term, decreased glucose uptake in the calves with diarrhea from BG and CG after diarrheic episodes may seem more logical, considering the damage caused by diarrhea to the epithelial cells. However, the changes caused by diarrhea in duodenal section presumably resulted from an intestinal adaptation to mitigate possible CHO malabsorption. Also, the glucose may be more locally utilized to support the adaptive increase in intestinal cell proliferation and repair at the time of recovery, after diarrhea has occurred.

Nevertheless, when we observe SLC5A1 mRNA levels in the calves without diarrhea we notice lowest expression in BG, probably because they have better duodenal CHO uptake than the CG. The greater demand of energy for weight gain and GIT development in BG may have significantly improved calves’ energy uptake and reduced glucose availability in this group in comparison to the others.

On the other hand, it is surprising that the plasma NEFA concentration was elevated in butyrate-fed calves. Since NEFA is released from the fat depot, it would be normal to think that the energy deficit was higher in the BG. However, we observed a tendency toward a higher ADG in this group in comparison to the CG. This fact led us to believe that, as butyrate is also a NEFA, when supplemented in the diet, it reaches the bloodstream, raising the levels of circulating NEFA. In this case, the increase would not be related to the mobilization of the fat reserve, which explains why BHB was not different between the groups. In addition, although differences between groups, the NEFA levels remained within the physiological levels (Yu et al., 2019).

The other metabolic parameters analyzed were also normal in all the groups, indicating that butyrate supplementation did not negatively affect mineral metabolism and the acid-base balance.

Concerning the growth of the animals, a tendency for more significant ADG was identified at 30 (P = 0.07) and 60 (P = 0.09) d of life, and a more substantial number of animals with twice the birth weight at 60 d of life was found in the BG group. In our study, the ADG rate found in both groups, considering the total preweaning period, is below the gains of 800 g/d found by Tümmler et al. (2021). However, the studies conducted in Brazil with calves fed with 6 L of milk per day (Slanzon et al., 2019; Weiller et al., 2021; Coelho et al., 2022) and data from the meta-analysis performed by Souza et al. (2016) showed ADG rates similar to those found in our study.

Studies are divergent on effects of butyrate on ADG. Kato et al. (2011), Araujo et al. (2015), Frieten et al. (2017), and Koch et al. (2019) found no difference in weight gain between butyrate-supplemented and non-
supplemented groups. However, Hill et al. (2007), Liu et al. (2021), and Guilloteau et al. (2009) observed more significant weight gain in animals supplemented with sodium butyrate. A point to be considered in these studies is the different types of liquid diets and the feeding levels fed to the calves. The results of Koch et al. (2019) shown that body growth at weaning was only enhanced by ad libitum milk replacer feeding and not by butyrate. In our study, butyrate was added to the whole milk. Whole milk is a complete composition and with adequate feeding volume can promote a superior performance compared with milk replacers (Coelho et al., 2022).

Our results are a trend, but they must be considered once they can be explained by the better use of nutrients, as well as of the greater development of ruminal papillae and intestinal villi, in addition to the lower occurrence of diarrhea, which possibly resulted in this tendency toward greater ADG.

Therefore, based on our results, sodium butyrate may be an interesting alternative to be used in the preweaning period of dairy calves, since it increased ruminal and intestinal development, reduced the days with abnormal stools, reduced the occurrence and severity of diarrhea.

CONCLUSIONS

Under the described experimental settings, the present findings demonstrated that supplementation with butyrate in the liquid diet improved the development and maturation of rumen and duodenum, and reduced morbidity, recurrence, and days with diarrheal feces in dairy calves in the preweaning period. These results indicate that supplementation with butyrate stimulates the development of the GIT and reduces the losses associated with diarrhea, reflecting a trend toward better ADG. Further studies evaluating the consumption of solid feeds, as well as with larger volumes of milk supplied daily to the calves can help to determine strategies for the different dairy systems existing in the world to produce healthy calves, whether providing more milk, supplementing the liquid diet with butyrate, or both.

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