

Effects of tributyrin supplementation in calf starter on growth, blood parameters and development of rumen and small intestine in Holstein calves fed milk replacers with different fatty acid profile.

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ABSTRACT

The objective of this study was to evaluate the effects of tributyrin (TB) supplementation in calf starter on development of the rumen and small intestine for calves fed two milk replacers differing in fatty acid composition. Fifty-four Holstein calves (38 heifers and 16 bull calves) were assigned to one of the four treatments: (1) milk replacer containing 31 g/kg C8:0 and 29 g/kg C10:0 in total fatty acids (MR-) and calf starter without TB (ST-) (MR-ST-; n = 14), (2) MR- and calf starter with a TB supplement (600 g/kg TB and 400 g/kg silicon dioxide) at 6 g/kg of DM (ST+) (MR-ST+; n = 13), (3) milk replacer containing 26 g/kg C4:0, 57 g/kg C8:0, and 56 g/kg C10:0 in total fatty acids (MR+) and ST- (MR+ST-; n = 13), and (4) MR+ and ST+ (MR+ST+; n = 14). Milk replacers were offered at 600 g/d (powder basis) from 8 to 14 d, increased up to 1,300 g/d from 15 to 21 d, 1,400 g/d from 22 to 49 d, decreased down to 700 g/d from 50 to 56 d, and 600 g/d from 57 to 63 d, and then weaned at 64 d of age. Data and samples were collected until 91 d of age. All the calves were fed one of the calf starters and chopped hay ad libitum from 8 d of age. Bull calves were euthanized at weaning to determine weights of digestive organs and evaluate ruminal and small intestinal morphology. Calf starter and milk replacer treatments did not affect dry matter intake, growth performance, digestive organs weights (kg/100kg of body weight), and rumen papillae length. However,

calves fed ST+ had greater ratio of villus height to crypt depth (2.20 vs. 1.84; $P < 0.01$) in the ileum compared to those fed ST-. In addition, calves fed MR+ had less crypt depth (240 vs. 298 μm ; $P = 0.02$) and greater ratio of villus height to crypt depth (2.22 vs. 1.91; $P < 0.01$) in the ileum compared to those fed MR-. A tendency for interaction between milk replacer and calf starter treatments was observed for the ratio of villus height to crypt depth in the jejunum, in which TB in calf starter increased the ratio only for calves fed MR-. Milk replacer treatment did not affect plasma glucagon-like peptide-2 (GLP-2) concentration. However, calves fed ST+ had greater plasma GLP-2 concentration compared to those fed ST- after weaning (1.16 vs. 0.80 ng/mL; $P < 0.02$). These results suggest that TB supplementation for dairy calves may affect small intestinal morphology at weaning, and increase plasma GLP-2 concentration after weaning.

Key words: butyrate, gastrointestinal tract, glucagon-like peptide-2

Abbreviations: ADG, average daily gain; AUC, area under the curve; BHB, β -hydroxybutyrate; BW, body weight; CP, crude protein; DM, dry matter; DMI, dry matter intake; GLP-2, glucagon-like peptide-2; MCFA, medium-chain fatty acids; NDF, neutral detergent fiber; PUFA, polyunsaturated fatty acids; TB, tributyrin; WSC, water soluble carbohydrate.

1. Introduction

Drastic changes in nutrient source and digestion process occur during the weaning transition in dairy calves. In dairy calves, gut health can have long-term effects on production. The number of days that calves have diarrhea during the first 4 months of life was negatively related to 305 d mature equivalent milk production during the first lactation (Heinrichs and Heinrichs, 2011). Thus, it is necessary to minimize digestive problems during the weaning transition by enhancing ruminal and small intestinal epithelial development.

Butyrate supplementation in calf starter has positive effects on development of the rumen and gastrointestinal tract in calves. In previous studies, sodium butyrate supplementation in calf starter increased papillae length in the rumen (Gorka et al., 2011a), and increased villus height in the jejunum and decreased crypt depth in the ileum (Gorka et al., 2014). Tributyrin (butanoic acid 1, 2, 3-propanetriyl ester; TB), triglyceride of butyrate, is a liquid at room temperature and does not have a butyrate-distinctive offensive odor, and can be a more practical supplement for dairy calves compared to sodium butyrate. Previously, we showed that TB supplementation in milk replacer increased plasma concentration of glucagon-like peptide-2 (GLP-2; Inabu et al., 2019), which stimulates intestinal growth (Taylor-Edwards et al., 2011). In addition, calves fed milk

replacer containing TB had greater total dry matter intake (DMI) after weaning, and greater body weight (BW) during the weaning transition and after weaning (Murayama et al., 2023). However, to our knowledge, effects of TB supplementation in calf starter were not investigated in dairy calves.

Previous studies showed that fatty acid profile in milk replacer affected the development of digestive organs and gut health in dairy calves (Guilloteau et al., 2009; Murayama et al., 2023), but interaction between butyrate supplementation in calf starter and fatty acid profile of milk replacer were not consistent in the literature. Gorka et al. (2011a) reported that sodium butyrate supplementation to milk replacer or calf starter enhanced rumen development in dairy calves, but that their effects were not additive. In contrast, addition of a fatty acid blend (butyrate, medium-chain fatty acids (MCFA), polyunsaturated fatty acids (PUFA)) to calf starter increased feed efficiency only when the fatty acid blend was added to milk replacer (Hill et al., 2011). These results suggest that the effects of butyrate supplementation in calf starter may vary depending on the type of milk replacer. In our previous study (Murayama et al., 2023), TB supplementation in milk replacer increased DMI and BW, and supplementation of caprylic acid (C8:0) and capric acid (C10:0) in milk replacer reduced incidence of diarrhea in dairy calves. The C8:0 and C10:0 have been reported to affect the intestinal microbiota and inhibit bacterial concentrations in in

vitro experiments (Zentek et al., 2011). As such, TB and MCFA supplementation in milk replacer might have affected the development of digestive organs and gut health, and would potentially affect animal responses to TB supplementation in calf starter.

We hypothesized that TB supplementation in calf starter change rumen and small intestinal morphology and TB and MCFA supplementation in milk replacer affect small intestinal morphology, which would have a synergistic effect on growth performance in dairy calves. Therefore, the objective of this study was to evaluate the effects of TB supplementation in calf starter on development of the rumen and small intestine in dairy calves fed two milk replacers differing in fatty acid composition.

2. Materials and methods

2.1. Animals and housing

All experimental procedures were approved by the Animal Care and Use Committee of Hiroshima University (# E20-2). Fifty-four Holstein calves (38 heifer and 16 bull calves), born between October 8 and November 18, 2020, were transported from commercial dairies to the Dairy Technology Research Institute (Yabuki, Fukushima, Japan) at 3-5 d age. When calves arrived at the research farm, they were checked for respiratory disease and diarrhea, and calves with clinical symptom were excluded from the study. Upon

arrival, calves received 5 mL of Terramycin (Zoetis Japan), 0.5 mL of Duphafral Forte (Zoetis Japan), 5 mL of Ivermectin PO (Fujita Pharm, Tokyo, Japan) via intramuscular injection, subcutaneous injection, and percutaneous absorption, respectively. In addition, calves received 5 mL of sulfonamides (Ektecin Liquid, Meiji Seika Pharma, Tokyo, Japan) daily for three consecutive days after arrival. The calves were fed 2 L of electrolyte solution on the day of arrival, and a milk replacer (280 g/kg crude protein (CP) and 180 g/kg fat) up to 600g/d (powder basis) thereafter until 8 d of age. At the beginning of the study (8 d of age), BW was 42.8 ± 3.60 kg, and serum immunoglobulin G concentration was 21.9 ± 14.93 g/L (mean \pm SD). The calves were raised outdoors in individual hutches (115 cm \times 230 cm \times 120 cm) bedded with sawdust on a rubber mat throughout the study.

2.2. Feeding

The calves were blocked by sex and balanced for birth date, BW, and farm origin and randomly assigned to one of the four treatments: (1) milk replacer containing 31 g/kg C8:0 and 29 g/kg C10:0 in total fatty acids (MR-) and calf starter without TB supplementation (MR-ST; 10 heifer and 4 bull calves), (2) MR- and calf starter with a TB supplement (600 g/kg TB and 400 g/kg silicon dioxide) at 6 g/kg on a dry matter

(DM) basis (MR-ST+; 9 heifer and 4 bull calves), (3) milk replacer containing 26 g/kg C4:0, 57 g/kg C8:0, 56 g/kg C10:0 in total fatty acids with addition of specific oils (MR+) and ST- (MR+ST-; 9 heifer and 4 bull calves), and (4) MR+ and ST+ (MR+ST+; 10 heifer and 4 bull calves). Experimental milk replacers (166.7g/L) were offered at 600 g/d (powder basis) from 8 to 14 d, increased up to 1,300 g/d from 15 to 21 d, 1,400 g/d from 22 to 49 d, decreased down to 700 g/d from 50 to 56 d, and 600 g/d from 57 to 63 d, and then weaned at 64 d of age. Data and samples were collected until 91 d of age. All the calves were fed one of the milk replacers using a bucket with a soft rubber nipple twice daily at 06:30 and 16:30 h, and one of the calf starters and chopped klein grass hay (102 g/kg CP and 668 g/kg neutral detergent fiber (NDF) on DM basis) ad libitum separately at 10:00 h from 8 days of age. Analyzed nutrient composition of the milk replacers are shown in Table 1. Ingredients and composition of the calf starters are shown in Table 2. Refused calf starter and hay were removed daily at 10:00 h, and their amounts were recorded.

2.3. Data and sample collection

Milk replacers, calf starters and hay were sampled monthly and stored at room temperature. Milk replacer, calf starter and hay intake of individual calves were recorded

daily. Fecal score (1-4 scale; 1 = normal consistency, 2 = semi-formed or pasty, 3 = loose feces and 4 = watery feces.; modified from the University of Wisconsin's calf health scoring chart) was recorded daily by two trained technicians and fecal scores 3 and 4 were determined as diarrhea. The days of diarrhea was defined as the number of days with fecal score ≥ 3 . Disease incidence, if any, was recorded daily. Body weight, withers height, hip height, horizontal body length, heart girth and hip width were measured (Sieber et al., 1988) at 09:30 h at the start of the trial (8 d of age) and weekly thereafter until the end of the trial (91 d of age). Blood samples were collected from the jugular vein at 10:00 h on 8 d of age and weekly thereafter, using evacuated tubes (Venoject II VP-H100K with heparin sodium; Terumo Corporation, Tokyo, Japan) for the collection of plasma. Immediately after sample collection, aprotinin (500 kallikrein inhibitor units/mL of blood; Sigma-Aldrich Inc., Tokyo, Japan) was added to the blood samples, centrifuged at $1,800 \times g$ at 4 °C for 20 min, and plasma was harvested. Plasma samples were stored at -80 °C until analysis. Bull calves were euthanized at 64 d of age. Calves were anesthetized (subcutaneous injection of Selactar 20 g/kg injection solution at 1.5 mL/100kg of BW; Bayer Yakuhin) and killed by exsanguination from the carotid artery. Ruminal and small intestinal tissues were sampled immediately after euthanasia. Rumen epithelial tissues (5 \times 5 cm) were sampled at the cranial ventral blind sac (Kato et al., 2011). Jejunum tissues

were sampled at 30 cm proximal to the collateral branch of the cranial mesenteric artery (Malmuthuge et al., 2015). Ileum tissues were sampled at 30 cm proximal to the ileo-cecal junction (Malmuthuge et al., 2015). Tissue samples were placed in PBS with 100 g/kg formalin immediately after collection, and processed into paraffin sections (4 μ m thick) as described by Nii et al. (2020). After tissue sampling, the reticulorumen, omasum, abomasum, small intestine, and large intestine were separated, emptied, rinsed repeatedly with water, drained, and weighed individually.

2.4. Sample analysis

The calf starter and hay samples were ground using a hammer mill (ST1; Retsch GmbH, Haan, Germany) with a 1-mm screen and analyzed by the ZEN-RAKU-REN Analysis Center (Kamisu, Ibaraki, Japan) for DM (method no. 930.15), ash (method no. 942.05), CP (method no. 990.03), fat (method no. 920.39), and starch (method no. 920.40) according to AOAC (1995), for NDF without heat-stable α -amylase according to Van Soest et al. (1991), and for water-soluble carbohydrate (WSC) concentration using anthrone assay according to Hall et al. (1999).

Plasma samples were analyzed for urea nitrogen, glucose, ketone body and GLP-2 concentrations. Plasma urea nitrogen, glucose and ketone body concentrations were

determined via enzymatic methods using a clinical chemistry analyzer (Bio Majesty TM, JACK-BM, JEOL Ltd., Akishima, Tokyo, Japan). Plasma GLP-2 concentration was measured using the time-resolved fluoroimmunoassay technique (Sugino et al., 2004, Inabu et al., 2017).

Tissue sections were stained with Hansen's hematoxylin and eosin for histometric analysis (Nii et al., 2020). Length of rumen papillae, villus height and crypt depth of small intestinal tissues were measured under a light microscope (Eclipse E400, Nikon, Tokyo, Japan) connected to image analysis software (NIS-Elements, Nikon, Tokyo, Japan).

2.5. Statistical Analysis

Two heifer calves had severe diarrhea at the beginning of the study, and were excluded from the study. Therefore, MR-ST+ and MR+ST- treatments had one calf less than the other treatments. The current study has sufficient power ($\alpha = 0.05$, power = 0.80) to detect a 15% difference in digestive organ and morphology variables and a 10% difference in the other variables. All data were screened for normality using JMP Pro 16 (SAS Institute Inc., Cary, NC, USA) and data that were not normally distributed were subjected to Box-Cox transformations to improve their normality before the statistical analyses. All data were analyzed as a 2×2 factorial arrangement of treatments involving two calf starters

(ST- vs. ST+) and two milk replacers (MR- vs. MR+). The DMI, growth performances measurements, fecal score, days of diarrhea and blood variables were analyzed separately for the 3 periods differing in the primary nutrient sources: 8–49 d (before weaning), 50–63 d (weaning transition), 64–91 d of age (after weaning) using JMP Pro 16 (SAS Institute Inc., Cary, NC, USA) with the following model:

$$Y_{ijklm} = \mu + M_i + S_j + W_k + X_l + MS_{ij} + MW_{ik} + SW_{jk} + MSW_{ijk} + MX_{il} + SX_{jl} + MSX_{ijl} + C_m + e_{ijklm},$$

Where Y_{ijklm} is the dependent variable, μ is the overall mean, M_i is the fixed effect of milk replacer treatment, S_j is the fixed effect of calf starter treatment, W_k is the fixed effect of week as a repeated measure, X_l is the fixed effect of sex and their interactions, C_m is the random effect of calves, and e_{ijklm} is the residual. Covariant structure of the model was compound symmetry. Initial BW, skeletal measures, blood variables were not different among the treatments, and they were not included in the model as covariate. Treatment by sex interactions were not significant for all response variables except for milk replacer intake and plasma urea nitrogen concentration before weaning. As such, results were not presented separately for male and female.

The digestive organ measurements were analyzed using the following model:

$$Y_{ijk} = \mu + M_i + S_j + MS_{ij} + C_k + e_{ijk},$$

where Y_{ijk} is the dependent variable, μ is the overall mean, M_i is the fixed effect of milk replacer treatment, S_j is the fixed effect of calf starter treatment, and their interactions, C_k is the random effect of calves, and e_{ijk} is the residual.

When significant $MR \times ST$ interactions or their tendencies were observed, difference among treatment means were evaluated with Tukey's test. Correlation coefficients (r_s) were determined between area under the curve (AUC) of plasma GLP-2 concentration from 8 to 63 d of age and villus height of the ileum using Spearman's correlation method using JMP Pro 16 (SAS Institute Inc., Cary, NC, USA). Significance was declared at $P < 0.05$, and tendency was declared at $0.05 \leq P < 0.10$.

3. Results

3.1. Intake, growth and fecal score

Calf starter and milk replacer treatments did not affect DMI throughout the study. Calves fed ST+ had greater C4:0 intake before weaning (3.3 vs. 3.1 g/d; $P < 0.01$), weaning transition (3.7 vs. 2.1 g/d; $P < 0.01$) and after weaning (4.9 vs. 0.0 g/d; $P < 0.01$). Calves fed MR+ had greater C4:0 intake before weaning (6.3 vs. 0.1 g/d; $P < 0.01$) and weaning transition (5.1 vs. 0.8 g/d; $P < 0.01$). Interaction effect between calf starter and milk replacer treatments was observed for C4:0 intake before weaning ($P < 0.01$) and weaning

transition ($P < 0.01$). Calves fed MR+ had greater C8:0 and C10:0 intakes before weaning (13.6 vs. 7.9 g/d; $P < 0.01$ and 13.4 vs. 7.4 g/d; $P < 0.01$, respectively) and weaning transition (9.2 vs. 5.3 g/d; $P < 0.01$ and 9.0 vs. 5.0 g/d; $P < 0.01$, respectively). Interaction effect between calf starter and milk replacer treatments was observed for hay intake before weaning ($P = 0.04$; Table 3), in which calves in MR-ST- treatment had higher hay intake compared to those in MR+ST- treatment (30 vs. 14 g/d; $P < 0.05$).

Calves fed ST+ tended to have greater withers height gain (0.22 vs. 0.20 cm/d; $P = 0.09$, Table 4) and hip height gain (0.22 vs. 0.20 cm/d; $P = 0.09$) before weaning compared to those fed ST-. Milk replacer treatment did not affect growth performance throughout the study. Fecal score and days of diarrhea were not affected by calf starter and milk replacer treatments throughout the study (Table 5).

3.2. Metabolites and hormones

Calf starter treatment did not affect plasma urea nitrogen and glucose concentrations throughout the experimental period (Table 6). Plasma GLP-2 concentration was also unaffected by calf starter treatment before weaning and during the weaning transition. However, after weaning, plasma GLP-2 concentration was higher for calves fed ST+ than those fed ST- (1.16 vs. 0.80 ng/mL; $P = 0.02$). Milk replacer treatment did not affect

plasma urea nitrogen, glucose and GLP-2 concentrations. Calves fed MR+ had greater plasma ketone body concentration compared to calves fed MR- before weaning (80.4 vs. 67.9 $\mu\text{mol/L}$; $P = 0.02$), but not during the weaning transition and after weaning.

3.3. Ruminal and intestinal morphology

Digestive organ weights (kg/100kg of BW) were not affected by milk replacer and calf starter treatments. However, calves fed ST+ tended to have lower omasum weight compared to those fed ST- (231 vs. 273 g; $P = 0.10$, Table 7). The other digestive organ weights were not affected by calf starter and milk replacer treatments. In addition, rumen papillae length, jejunum villus height and crypt depth were unaffected by calf starter and milk replacer treatments. A tendency for interaction between calf starter and milk replacer treatments was observed for the ratio of villus height to crypt depth in the jejunum ($P = 0.06$), in which calves in MR-ST+ treatment had higher ratio of villus height/crypt depth compared to those of MR-ST- treatment (2.27 vs. 1.89; $P = 0.05$) while this was not observed in MR+ treatments. In the ileum, villus height was not affected by calf starter and milk replacer treatments. However, ST+ tended to decrease crypt depth in the ileum compared to ST- (248 vs. 290 μm ; $P = 0.08$), and MR+ decreased crypt depth in the ileum compared to MR- (240 vs. 298 μm ; $P = 0.02$). Furthermore, the ratio of villus height to

crypt depth was higher in calves fed ST+ and MR+ calves compared to ST- and MR- calves (2.30 and 1.84; $P < 0.01$, and 2.22 vs. 1.91; $P < 0.01$, respectively). Positive correlation was observed between villus height in the ileum and area under the curve (AUC) of plasma GLP-2 concentration before weaning ($r_s = 0.54$; $P = 0.04$; Figure 1).

4. Discussion

4.1. Effects of TB in calf starter

Butyrate is a stimulator of ruminal and intestinal development (Gorka et al., 2018). One of the aims of feeding TB in calf starter (ST+) was to increase rumen papillae length. In a previous study, sodium butyrate supplementation in calf starter increased rumen papillae length (Gorka et al., 2011a). However, rumen papillae length did not increase in calves fed ST+ in this study. We supplemented butyrate to calf starter as TB, which is triglyceride of butyrate, and butyrate is released in the rumen after TB is hydrolyzed by microbial lipases. Mallo et al. (2012) reported that the amount of butyrate released from monobutyrate after 7 hours of incubation in vitro was only 359 g/kg of that of fat-encapsulated sodium butyrate. We did not evaluate the release of butyrate from the TB in rumen in the current study. However, TB is glycerol esters of butyrate, and it is possible that limited release of butyrate from the TB might have reduced its action in the rumen,

allowing some butyrate to reach the small intestine and exert its effect there.

Feeding ST+ increased the ratio of villus height to crypt depth in the ileum regardless of the type of milk replacers, and it is partly attributed to a tendency of decreased crypt depth. Our results are consistent with Gorka et al. (2014) who reported that butyrate supplementation in calf starter decreased crypt depth, and increased the ratio of villus height to crypt depth in the ileum of calves. Butyrate enhances proliferation, differentiation, and maturation of epithelial cells, and expedites regeneration (Guilloteau et al., 2010). As greater crypt depth or crypt hyperplasia indicates a reparative or regenerative process (Serra and Jani, 2007), the tendency for reduced crypt depth for ST+ at the time of sampling, immediately after completion of weaning, may suggest that butyrate reduced epithelial damages during weaning or expedited reparative processes from insults.

The intestinal morphology and function change during the weaning transition. In piglets, the change from liquid feed to solid feed during the weaning transition altered intestinal morphology and negatively affected its function (van Beers-Schreurs et al., 1998, Tang et al., 2022), which causes diarrhea (Heo et al., 2013). Their villus height and the ratio of villus height to crypt depth decreased, and crypt depth increased in the small intestine during the weaning transition (Tang et al., 1999, Hu et al., 2013, Bomba et al., 2014).

Villus atrophy and crypt hyperplasia that occurred during the weaning transition may indicate that the balance between intestinal cell proliferation and apoptosis was disrupted (Montagne et al., 2007) and that the activity and digestion capacity of disaccharidase decreased (Pluske et al., 1997, Tang et al., 2022). Therefore, the greater ratio of villus height to crypt depth, observed for ST+ treatments in the current study, may indicate that digestion and absorption capacity of nutrients were maintained during the weaning transition. However, we did not evaluate treatment effects on nutrient digestibility, and further research is needed to evaluate whether TB supplementation affects nutrient digestibility.

We had hypothesized that feeding TB in calf starter would increase villus height in the small intestine, and that its effect would be mediated by GLP-2. In a previous study, sodium butyrate supplementation in calf starter increased GLP-2 secretion (Gorka et al., 2009). In addition, intraruminal infusions of sodium butyrate increased plasma GLP-2 concentrations in sheep (Elsabagh et al., 2017). Taylor-Edwards et al. (2011) reported that subcutaneous injection of GLP-2 for 10 days increased villus height in small intestine of calves. However, in the current study, feeding TB in calf starter did not increase plasma GLP-2 concentration before weaning nor villus height in the jejunum or ileum at weaning, which is possibly due to variable or insufficient TB intake from calf starter before

weaning. However, AUC of plasma GLP-2 concentration before weaning was positively correlated with ileal villus height at weaning in the current study, and we cannot exclude a possible role of GLP-2 affecting villus height. As the ST+ treatment increased plasma GLP-2 concentration after weaning, its effects on intestinal morphology and development during the post-weaning period should be evaluated in future studies.

As described above, ST+ caused morphological changes, in the ileum, that are potentially beneficial during the weaning transition. In previous studies, including a blend of fatty acids (butyrate, coconut oil and flax oil) in calf starter increased total-tract digestibility, feed efficiency and average daily gain (ADG) (Hill et al., 2011, Quigley et al., 2019). However, there were no differences in DMI, feed efficiency, and growth performance among the treatments throughout the current study. When butyrate is used in calf starter, its positive effects on DMI and growth performance were shown with supplemental doses ranging from 3 to over 10 g/kg of DM (Gorka et al., 2018). In the current study, we added TB aiming to get 3 g/kg butyrate on a DM basis in the calf starter. However, ST+ contained 2 g/kg butyrate on a DM basis as measured, which may have been insufficient to improve growth performance in calves.

4.2. Effects of milk replacer

We found that feeding MR⁺ decreased crypt depth, and increased the ratio of villus height to crypt depth in the ileum, which is consistent with findings of Gorka et al. (2014) and Koch et al. (2019). In addition, a tendency of interaction effect between calf starter and milk replacer treatments was observed for the ratio of villus height to crypt depth in the jejunum; feeding TB in calf starter increased the ratio of villus height/crypt depth only for calves fed MR⁻. Similar to our results, Gorka et al. (2014) found the positive effect of butyrate in calf starter on the ratio of villus height to crypt depth in the ileum only for calves fed milk replacer without butyrate. These data collectively suggest that butyrate fed with milk replacer can affect morphology of the small intestine, and that providing additional butyrate from calf starter has less marginal effects. In this study, we observed another interaction effect between calf starter and milk replacer treatments for hay intake before weaning, in which MR⁺ decreased hay intake only for calves fed ST⁻. However, pre-weaned calves consumed small amounts of hay, and its biological implications are unknown and limited if any.

Evaluation of milk replacer treatment was not the primary objective of the current study, but calves fed MR⁺ had greater plasma ketone body concentration before weaning compared to MR⁻ calves. Quigley et al., (1991) reported that blood β -hydroxybutyrate (BHB) concentration was an indicator of rumen development in calves. In addition, serum

BHB concentration was positively correlated to the level of solid feed intake in dairy calves (Inabu et al., 2017). However, solid feed intake during the pre-weaning period and rumen papillae length were not affected by milk replacer treatment in this study. These results suggested that the increased BHB concentration resulting from MR+ feeding is not due to increased metabolic activity and development of the rumen epithelium. Previous study showed that calves fed coconut oil (rich of medium-chain fatty acids) had higher production of ketone body in liver slices than calves fed beef tallow (Graulet et al., 2000). In the current study, calves fed MR+ had 1.7 times higher C8:0 and C10:0 intakes. Therefore, we cannot exclude the possibility that the increase of plasma ketone body concentration by feeding MR+ was due to differences of MCFA intake. On the other hand, in our previous study (Murayama et al., 2023), serum BHB concentration before weaning was increased by supplementation of MCFA, as well as TB, in milk replacer. Based on these results, greater plasma ketone body concentration for calves fed MR+ can be attributed to both TB and MCFA.

5. Conclusions

Feeding TB in calf starter did not affect DMI, growth performance and rumen development, but increased the ratio of villus height to crypt depth in the ileum at weaning

regardless of milk replacer type. In addition, TB supplementation in calf starter increased plasma GLP-2 concentration after weaning. Further research is warranted about its effects on nutrient digestibility, gut development, and its morphology during the post-weaning period.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

The authors thanks Chika Nii of Hiroshima University and staffs of Dairy Technology Research Institute of ZEN-RAKU-REN for their technical assistance in sample collection and analysis. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors have stated no conflicts of interest.

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Table 1. Nutrient composition of milk replacers.

Item	Milk replacer	
	MR-	MR+
DM, g/kg	965	957
Nutrient composition, g/kg of DM		
CP	295	300
Fat	222	209
NFC	408	416
WSC ^a	342	353
Fatty acids, g/kg in total fatty acid		
C4:0	ND ^b	26
C6:0	1	1
C8:0	31	57
C10:0	29	56
C12:0	112	109
Other SFA	386	354
Other UFA	442	396

^aWater-soluble carbohydrate.

^bNot detected.

547 Table 2. Ingredient composition and nutrient composition of calf starters.

Item	Calf starter	
	ST-	ST+
Ingredient, g/kg of DM		
Steam-flaked barley grain	202	202
Steam-flaked corn grain	99	99
Alfalfa dehydrated pellet	37	7
Molasses cane	4	4
Pellet	658	658
Pellet, g/kg of DM		
Soybean meal	173	171
Dry ground corn	149	148
Wheat bran	90	89
Heated soybean ^a	71	70
Ground beet pulp	41	41
Corn gluten meal	23	23
Soybean flour	22	22
Wheat feed flour	16	16
Rapeseed meal	13	12
Cane molasses	33	33
Calcium carbonate	12	12
Salt	7	7
Calcium phosphate	6	6
Premix of trace minerals and vitamins ^b	4	4
Tributylin supplement ^c	-	6
DM, g/kg	887	887
Nutrient composition, g/kg of DM		
CP	229	230
Fat	37	39
Ash	67	71
NDF	183	175
Starch	327	332
Butyrate	ND ^d	2

548 ^aHeated Soybean meal (Soy plus, West Central Cooperative, Ralston, IA).

549 ^bContained 2,905 kIU/kg vitamin A, 600 kIU vitamin D, 29,800 mg/kg vitamin E,
550 1,200mg/kg thiamin, 1,200 mg/kg riboflavin, 2,400 mg/kg pantothenic acid, 4,780
551 mg/kg niacin, 720 mg/kg pyridoxin, 24 mg/kg biotin, 50 mg/kg folate, 35,720 mg/kg
552 choline, 5 mg/kg vitamin B₁₂, 1,905 mg/kg Fe, 953 mg/kg Cu, 3,810 mg/kg Zn,
553 3,810mg/kg Mn, 10 mg/kg Co and 48 mg/kg I.
554 ^cContained 600 g/kg tributyrin and 400 g/kg silicon dioxide.
555 ^dNot detected.
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Table 3. Effects of tributyrin supplementation in milk replacer and calf starter on DMI before weaning, weaning transition and after weaning; LSM \pm SEM

Item	Treatment ¹				SEM	<i>P</i> -value		
	MR-ST-	MR-ST+	MR+ST-	MR+ST		MR	ST	MR×ST
	(n = 14)	(n = 13)	(n = 13)	+ (n = 14)				
Before weaning (8-49 d of age)								
Milk replacer	1146	1147	1148	1138	4.6	0.18	0.35	0.24
Calf starter	117	120	128	122	26.1	0.49	0.95	0.49
Hay	30 ^a	17 ^{ab}	14 ^b	19 ^{ab}	4.2	0.10	0.35	0.04
Total DMI ²	1295	1285	1291	1279	27.8	0.98	0.83	0.76
C4:0 ³	< 0.01 _c	0.2 ^b	6.2 ^a	6.4 ^a	0.03	< 0.01	< 0.01	< 0.01
C8:0 ⁴	7.9	7.9	13.7	13.6	0.04	< 0.01	0.31	0.23
C10:0 ⁴	7.4	7.4	13.4	13.3	0.04	< 0.01	0.31	0.24
Weaning transition (50-63 d of age)								
Milk replacer	768	772	769	769	2.2	0.48	0.44	0.44
Calf starter	1071	919	1045	1098	102.2	0.62	0.91	0.34
Hay	153	136	95	138	22.8	0.22	0.58	0.20
Total DMI	1994	1827	1911	2003	101.8	0.65	0.83	0.26
C4:0 ³	< 0.0 _{1^d}	1.5 ^c	4.2 ^b	6.0 ^a	0.10	< 0.01	< 0.01	< 0.01
C8:0 ⁴	5.3	5.3	9.2	9.2	0.01	< 0.01	0.44	0.44
C10:0 ⁴	4.9	5.0	9.0	9.0	0.01	< 0.01	0.44	0.44
After weaning (64-91 d of age)								
Calf starter	3033	2833	2975	3125	120.5	0.27	0.75	0.13
Hay	188	166	186	202	39.6	0.67	0.94	0.62
Total DMI	3221	3000	3161	3325	121.5	0.23	0.70	0.12
C4:0 ³	< 0.0 ₁	4.6	< 0.0 ₁	5.1	0.14	0.10	< 0.01	0.10

^{a,b,c,d}Least squares means within a row with different superscripts differ ($P < 0.05$)

¹ MR- = milk replacer containing 31 g/kg C8:0 and 29 g/kg C10:0 in total fatty acids

without tributyrin supplementation; MR+ = milk replacer containing 57 g/kg C8:0 and 56 g/kg C10:0 in total fatty acids with 6 g/kg (DM basis) tributyrin supplementation; ST- = calf starter without tributyrin supplementation; ST+ = calf starter with 6 g/kg (DM basis) tributyrin supplement (contained 600 g/kg tributyrin and 400 g/kg silicon dioxide).

²Total DMI was the sum of milk replacer, calf starter and hay.

³Calculated based on milk replacer and calf starter intake.

⁴Calculated based on milk replacer intake.

580 Table 4. Effects of tributyrin supplementation in milk replacer and calf starter on growth

581 performance before weaning, weaning transition and after weaning; LSM \pm SEM

Item	Treatment ^a				SEM	P-value		
	MR-ST- (n = 14)	MR-ST+ (n = 13)	MR+ST- (n = 13)	MR+ST+ (n = 14)		MR	ST	MR×ST
Before weaning (8-49 d of age)								
ADG, kg/d	0.97	0.95	0.95	0.95	0.029	0.68	0.45	0.74
Withers height	0.21	0.23	0.18	0.22	0.016	0.17	0.09	0.46
gain, cm/d								
Hip height	0.21	0.23	0.19	0.22	0.016	0.44	0.09	0.72
gain, cm/d								
Heart girth	0.44	0.45	0.48	0.46	0.022	0.25	0.77	0.51
gain, cm/d								
Horizontal	0.31	0.30	0.31	0.30	0.019	0.94	0.60	0.86
body length								
gain, cm/d								
Hip width gain,	0.11	0.10	0.11	0.10	0.021	0.60	0.14	0.98
cm/d								
Feed	0.73	0.72	0.69	0.72	0.022	0.48	0.75	0.41
efficiency ^b								
Weaning transition (50-63 d of age)								
ADG, kg/d	0.95	0.92	1.01	1.01	0.068	0.24	0.58	0.99
Withers height	0.27	0.21	0.28	0.26	0.037	0.40	0.29	0.63
gain, cm/d								
Hip height	0.27	0.25	0.32	0.26	0.038	0.43	0.31	0.67
gain, cm/d								
Heart girth	0.36	0.28	0.34	0.35	0.052	0.68	0.52	0.40
gain, cm/d								
Horizontal	0.29	0.32	0.32	0.33	0.057	0.70	0.70	0.82
body length								
gain, cm/d								
Hip width gain,	0.11	0.13	0.10	0.10	0.016	0.16	0.46	0.60
cm/d								
Feed efficiency	0.47	0.50	0.51	0.50	0.026	0.38	0.76	0.46

After weaning (64-91 d of age)

ADG, kg/d	1.21	1.17	1.22	1.22	0.050	0.67	0.87	0.69
Withers height	0.25	0.23	0.26	0.22	0.022	0.92	0.23	0.64
gain, cm/d								
Hip height	0.25	0.21	0.23	0.28	0.024	0.24	0.99	0.07
gain, cm/d								
Heart girth	0.39	0.40	0.36	0.42	0.031	0.92	0.31	0.44
gain, cm/d								
Horizontal	0.32	0.28	0.31	0.29	0.030	0.91	0.31	0.74
body length								
gain, cm/d								
Hip width gain,	0.09	0.09	0.09	0.10	0.012	0.63	0.85	0.58
cm/d								
Feed efficiency	0.38	0.38	0.39	0.37	0.022	0.86	0.68	0.65

^a MR- = milk replacer containing 31 g/kg C8:0 and 29 g/kg C10:0 in total fatty acids without tributyrin supplementation; MR+ = milk replacer containing 57 g/kg C8:0 and 56 g/kg C10:0 in total fatty acids with 6 g/kg (DM basis) tributyrin supplementation; ST- = calf starter without tributyrin supplementation; ST+ = calf starter with 6 g/kg (DM basis) tributyrin supplement (contained 600 g/kg tributyrin and 400 g/kg silicon dioxide).

^bFeed efficiency was calculated to as the ratio of ADG (kg/d) to total DMI (kg/d).

Table 5. Effects of tributyrin supplementation in milk replacer and calf starter on fecal score and days of diarrhea before weaning, weaning transition and after weaning; LSM \pm SEM

Item	Treatment ^a				SEM	P-value		
	MR-ST- (n = 14)	MR-ST+ (n = 13)	MR+ST- (n = 13)	MR+ST+ (n = 14)		MR	ST	MR×ST
Before weaning (8-49 d of age)								
Fecal score	1.9	1.9	1.9	2.0	0.10	0.42	0.23	0.23
Days of diarrhea ^b	10.2	10.5	10.2	13.1	1.53	0.62	0.49	0.84
Weaning transition (50-63 d of age)								
Fecal score	1.6	1.5	1.3	1.4	0.13	0.22	0.63	0.88
Days of diarrhea ^b	2.0	1.5	0.9	0.7	0.68	0.23	0.74	0.40
After weaning (64-91 d of age)								
Fecal score	1.8	1.6	1.8	1.8	0.14	0.90	0.33	0.49
Days of diarrhea ^b	5.8	2.8	4.7	4.5	1.40	0.89	0.71	0.70

^aMR- = milk replacer containing 31 g/kg C8:0 and 29 g/kg C10:0 in total fatty acids without tributyrin supplementation; MR+ = milk replacer containing 57 g/kg C8:0 and 56 g/kg C10:0 in total fatty acids with 6 g/kg (DM basis) tributyrin supplementation; ST- = calf starter without tributyrin supplementation; ST+ = calf starter with 6 g/kg (DM basis) tributyrin supplement (contained 600 g/kg tributyrin and 400 g/kg silicon dioxide).

^bDiarrhea: fecal scores 3 and 4.

Table 6. Effects of tributyrin supplementation in milk replacer and calf starter on plasma metabolites and hormones before weaning, weaning transition and after weaning; LSM \pm SEM

Item	Treatment ^a				SEM	<i>P</i> -value		
	MR-ST- (n = 14)	MR-ST+ (n = 13)	MR+ST- (n = 13)	MR+ST+ (n = 14)		MR	ST	MR×ST
Before weaning (8-49 d of age)								
Urea nitrogen, mg/dL	10.6	10.0	10.3	10.7	0.41	0.64	0.80	0.26
Glucose, mg/dL	113.7	113.3	111.3	112.7	2.46	0.55	0.83	0.72
Ketone body, μmol/L	67.6	68.2	74.6	86.2	5.15	0.02	0.24	0.29
GLP-2 ^b , ng/mL	1.14	1.17	1.00	1.21	0.145	0.60	0.24	0.46
Weaning transition (50-63 d of age)								
Urea nitrogen, mg/dL	12.1	10.9	11.5	11.6	11.52	1.00	0.29	0.24
Glucose, mg/dL	111.7	113.0	112.9	112.1	2.76	0.96	0.92	0.72
Ketone body, μmol/L	196.8	167.2	178.9	183.5	15.53	0.96	0.42	0.28
GLP-2, ng/mL	1.01	0.99	0.97	1.21	0.166	0.94	0.56	0.47
After weaning (64-91 d of age)								
Urea nitrogen, mg/dL	12.8	12.3	13.3	12.7	0.65	0.48	0.46	0.95
Glucose, mg/dL	107.2	104.5	106.7	106.2	2.33	0.81	0.49	0.63
Ketone body, μmol/L	343.8	380.5	289.9	346.2	31.34	0.17	0.15	0.76
GLP-2, ng/mL	0.83	0.97	0.77	1.34	0.152	0.92	< 0.01	0.13

^aMR- = milk replacer containing 31 g/kg C8:0 and 29 g/kg C10:0 in total fatty acids

611 without tributyrin supplementation; MR+ = milk replacer containing 57 g/kg C8:0 and 56
612 g/kg C10:0 in total fatty acids with 6 g/kg (DM basis) tributyrin supplementation; ST- =
613 calf starter without tributyrin supplementation; ST+ = calf starter with 6 g/kg (DM basis)
614 tributyrin supplement (contained 600 g/kg tributyrin and 400 g/kg silicon dioxide).
615 ^bGlucagon-like peptide-2
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Table 7. Effects of tributyrin supplementation in milk replacer and calf starter on rumen and gastrointestinal weight, papillae length, villus height, crypt depth and ratio of villus height/crypt depth in jejunum and ileum at weaning in bull calves; LSM \pm SEM

Item	Treatment ^a				SEM	<i>P</i> -value		
	MR-ST- (n = 4)	MR-ST+ (n = 4)	MR+ST- (n = 4)	MR+ST+ (n = 4)		MR	ST	MR×ST
Weight, g								
Reticulorumen	1240	1090	1201	1097	104.8	0.88	0.25	0.83
Omasum	279	237	266	225	22.9	0.59	0.10	1.00
Abomasum	459	418	452	483	21.3	0.19	0.83	0.12
Small intestine	3265	3116	3106	2812	233.2	0.34	0.36	0.76
Large intestine	776	706	804	762	48.7	0.41	0.27	0.79
Weight, kg/100 kg of BW								
Reticulorumen	1.20	1.08	1.13	1.09	0.085	0.75	0.36	0.68
Omasum	0.27	0.24	0.25	0.22	0.018	0.41	0.12	0.91
Abomasum	0.44	0.42	0.42	0.48	0.025	0.42	0.56	0.13
Small intestine	3.14	3.11	2.91	2.78	0.179	0.15	0.66	0.79
Large intestine	0.75	0.71	0.76	0.75	0.057	0.63	0.67	0.75
Rumen papillae length, mm	4.61	5.59	5.34	6.02	0.484	0.26	0.12	0.76
Jejunum								
Villus height, μm	690	790	775	983	105.1	0.21	0.17	0.62
Crypt depth, μm	365	349	354	458	51.3	0.36	0.41	0.27
Villus height/crypt depth	1.89 ^b	2.27 ^a	2.19 ^{ab}	2.18 ^{ab}	0.090	0.28	0.07	0.06
Ileum								
Villus height, μm	542	577	500	548	33.1	0.31	0.24	0.83
Crypt depth, μm	329	268	250	229	21.3	0.02	0.08	0.37
Villus height/crypt depth	1.67	2.16	2.00	2.44	0.084	< 0.01	< 0.01	0.75

^{a,b}Least squares means within a row with different superscripts differ ($P < 0.05$)

¹MR- = milk replacer containing 31 g/kg C8:0 and 29 g/kg C10:0 in total fatty acids

623 without tributyrin supplementation; MR+ = milk replacer containing 57 g/kg C8:0 and 56
624 g/kg C10:0 in total fatty acids with 6 g/kg (DM basis) tributyrin supplementation; ST- =
625 calf starter without tributyrin supplementation; ST+ = calf starter with 6 g/kg (DM basis)
626 tributyrin supplement (contained 600 g/kg tributyrin and 400 g/kg silicon dioxide).

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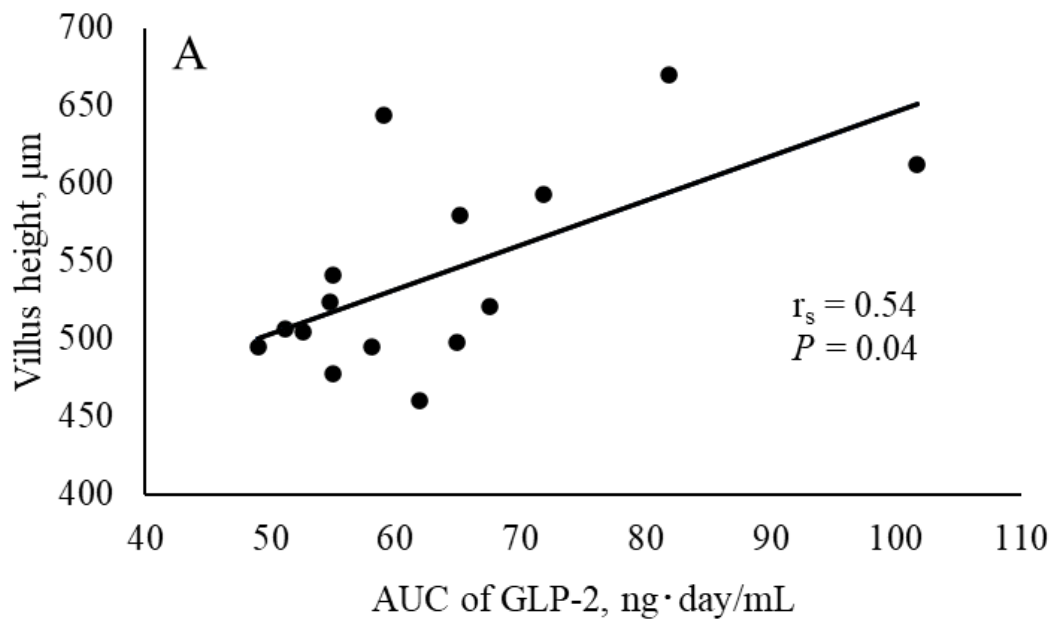
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642 Figure 1. Spearman's correlation coefficient (r_s) between AUC of plasma glucagon-like
 643 peptide-2 (GLP-2) concentration from 8 to 63 d of age and ileal villus height at weaning
 644 in bull calves ($n = 16$).