Animal Nutrition 10 (2022) 399–411

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Tributyrin administration improves intestinal development and health in pre-weaned dairy calves fed milk replacer

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ARTICLE INFO

Article history:
Received 10 August 2021
Received in revised form
28 March 2022
Accepted 14 June 2022
Available online 21 June 2022

Keywords:
Dairy calves
Tributyrin
Gut microbiota
Inflammation
Barrier function

ABSTRACT

Butyrate and its derivatives possess various nutritional and biological benefits for mammals, whereas its effects on dairy calves have not been well characterized. This study evaluated the effects of tributyrin administration on blood immune, intestinal immune and barrier functions, and microbial composition of pre-weaned dairy calves. Twenty newborn Holstein bull calves were randomly assigned into a control group (no tributyrin supplementation, CON; $n = 10$) or a treatment group (supplemented with tributyrin at 2 g/L of milk, TRB; $n = 10$). The results showed that diarrhea frequency was decreased significantly by tributyrin administration from d 29 to 56 ($P < 0.001$) and the whole period ($P = 0.003$, d 1 to 56) though no significant effects were observed on growth performance. For blood metabolites, tributyrin administration significantly reduced the concentration of interleukin-1β (*IL-1β*) on d 28 ($P = 0.001$) and tended to reduce the concentration of serum amyloid A on d 56 ($P = 0.079$), whereas serum oxidative status parameters were not affected. For intestinal development, tributyrin administration increased the villus height ($P < 0.001$) and the ratio of villus height to crypt depth ($P = 0.046$) in the jejunum, and the villus height in the ileum ($P = 0.074$). Furthermore, toll-like receptor 2 (*TRL2*, $P = 0.045$) and *IL-1β* ($P = 0.088$) gene expressions were downregulated, while claudin-4 ($P = 0.022$) gene expression was upregulated in the jejunum following tributyrin administration. In the ileum, claudin-4 ($P = 0.029$) and G-protein coupled receptor 41 ($P = 0.019$) gene expressions were upregulated in the TRB group compared to CON. No significantly higher abundances of microbiota were found in the jejunum or ileum of calves in the CON group. In the TRB group, supplementing tributyrin significantly increased the abundance of short-chain fatty acid (SCFA)-producing bacteria, including Ruminococcaceae, Lachnospiraceae, *Prevotella* and Rikenellaceae (LDA >3.5, $P < 0.05$), which was negatively associated with inflammatory gene expression (*TLR2* and *IL-1β*) but positively associated with intestinal barrier genes (claudin-4) and morphological parameters ($P < 0.05$). In conclusion, supplementing tributyrin in milk replacer could improve intestinal

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2022.06.004

development and health of pre-weaned dairy calves by stimulating SCFA-producing bacteria colonization, enhancing intestinal barrier functions and suppressing inflammatory responses.

1. Introduction

Short-chain fatty acids (SCFAs), the main metabolites produced by bacterial fermentation of indigestible foods in the gastrointestinal tract (GIT), can directly activate G-coupled-receptors, inhibit histone deacetylases, and serve as energy substrates (Koh et al., 2016). They therefore have important metabolic functions and are crucial to intestinal health. The most abundant SCFAs in the gut are acetate, propionate and butyrate, among which butyrate has received particular attention due to its beneficial effects on intestinal homeostasis (Guilloteau et al., 2010a). Previous studies have shown that butyrate is indispensable in modulating intestinal mucosal immunity, inflammatory responses and intestinal barrier functions (Tan et al., 2014; Yang et al., 2020).

Calves in the pre-weaning phase are highly susceptible to disease and death. According to a recent USA survey, about 30% of the deaths in pre-weaned calves were caused by digestive diseases (Urie et al., 2018), which highlights the importance of improving gut health. Butyrate has been supplemented in feed as butyrate salts (calcium, sodium, potassium, or magnesium) or butyrins to improve the health and growth performance of dairy calves (Bedford and Gong 2018; Górka et al., 2018). Most of the studies conducted so far have mainly characterized the effects of butyrate on the efficiency of nutrient digestion (Guilloteau et al., 2010b), intestinal morphological functions (Gorka et al., 2011; Kato et al., 2011), and the activity of digestive enzymes (Gorka et al., 2014; Guilloteau et al., 2009) of dairy calves. Several studies in piglets and poultry showed that butyrate supplementation could improve tight junction protein expression of intestinal mucosa, decrease malondialdehyde (MDA), tumor necrosis factor a (TNF-a), interleukin-6 (IL-6), and interleukin-1β (IL-1β), and increased interleukin-10 (IL-10) concentrations in serum or intestinal mucosa (Bortoluzzi et al., 2017; Huang et al., 2015; Zou et al., 2019). However, few attempts have been made to understand how exogenous butyrate affects intestinal mucosal immunity, inflammatory responses and intestinal barrier function of pre-weaned dairy calves. Moreover, intestinal microbes play an important role in host immune system development, metabolism, and health (Arrieta et al., 2014; Subramanian et al., 2015). Some studies documented that butyrate administration could modify the structure, composition and function of the microbiota in poultry and piglets (Bortoluzzi et al., 2017; Sun et al., 2020). So far, few studies reported the effects of butyrate on the microbiota of dairy calves. O'Hara et al. (2018) demonstrated that calves supplemented with butyrate-fortified milk replacer (MR) improved the microbiota community. However, how intestinal microbiota of calves supplemented with butyrate affect intestinal immune and barrier function has not been well characterized.

Tributyrin (butanoic acid 1,2,3-propanetriyl ester), a source of butyrate, has no unpleasant smell compared to butyrate salts. Compared to butyrate salts, tributyrin is more efficacious, directly acts on the cells, has a longer plasma half-life of butyrate following oral delivery, and exhibits more favorable pharmacokinetics (Conley et al., 1998; Newmark et al., 1994). In this study, we aimed to investigate the effects of tributyrin oral delivery on growth performance, blood and intestinal immunity, and intestinal barrier function of pre-weaned dairy calves. Furthermore, we evaluated the effects of tributyrin on small intestinal microbiota and the relationship between microbiota and immune-related parameters. We hypothesized that tributyrin would improve intestinal development and health, and alter the diversity of gut microflora of pre-weaned dairy calves.

2. Materials and methods

The animal experiment was conducted according to the Regulation of the Administration of Laboratory Animals (2017 Revision) promulgated by Decree No. 676 of the State Council, China. The animal care protocol was approved by the Animal Care and Use Committee of China Agricultural University (Beijing, China), and all efforts were made to minimize animal suffering.

2.1. Animals and treatments

We collected and assigned the animals based on a randomized complete block design according to birth date. Twenty newborn Holstein bull calves (birth weight = 40.7 ± 2.10 kg; mean ± SD) from the Dairy Teaching and Research Farm (Beijing, China) were divided into 10 blocks of 2 calves (age difference within 2 d). Each block of animals was randomly assigned into a control (CON) or a treatment (TRB) group (Fig. 1). Calves in CON group were fed MR with no tributyrin supplementation ($n = 10$), while calves in TRB group were fed MR supplemented with tributyrin products (unprotected solid powder [35:65, tributyrin:silicon dioxide] provided by Guangdong VTR Biotechnology Co., Ltd, Guangdong, China) at 2 g/L (containing 35% pure tributyrin) of MR ($n = 10$). All the calves had successful passive transfer of immunity (serum total protein ≥5.5 g/dL [Tyler et al., 1996]).

2.2. Housing and feeding

Each calf received 4 L of pasteurized colostrum (evaluated by a Brix refractometer, > 22% (Quigley et al., 2013)) using a 4-L esophageal tubing feeding bottle within 1 h after birth. From d 2 to 56, the calves were housed in individual hutches (1.5 m × 2.0 m; width × length) surrounded by iron (coated by polyvinyl chloride) bar fences. The distance between hutches was approximately 80 cm, so that calves could hear and see other calves in neighboring hutches but could not have nose-to-nose or mouth-to-mouth contact. Each hutch was bedded with straw, which was changed weekly. The MR was mixed in a ratio of 1:7 with warm water (about 42 °C) and fed buckets (without teats). No antibiotics, probiotics, prebiotics or butyrate were included in the MR (FrieslandCampina Co., Ltd, Amersfoort, Netherlands). Calves were fed MR twice per day at 07:00 and 15:00 with 4 L/d from d 2 to 3, 6 L/d from d 4 to 10, and 8 L/d from d 11 to 42. Weaning was carried out by reducing milk volume from d 43. Calves were fed 6 L/d from d 43 to 49 and 4 L/d from d 50 to 56. For all TRB calves, commercial tributyrin products were added to the MR manually and mixed vigorously before immediately feeding the calves. Calves had free access to water and pelleted starter from d 3. All feeding implements and facilities were cleaned daily.

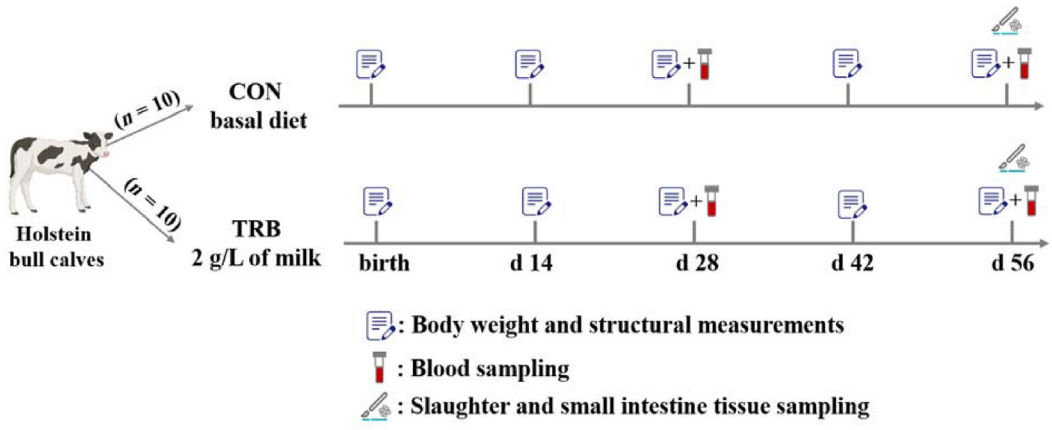

Fig. 1. Diagram of the experimental design. CON = control, supplemented with no tributyrin; TRB = tributyrin, supplemented with 2 g/L of milk.

Table 1
Chemical composition of experimental feeds (on DM basis).

Nutrient composition[1], %	Milk replacer	Starter[2]
DM	96.0	89.5
CP	22.0	19.5
Ether extract	20.0	2.79
Lactose	39.7	–
Starch	–	29.1
Crude ash	9.0	8.15
NDF	–	21.8
ADF	–	6.77

[1] The nutritive values are the means of the results of the analysis of samples collected each week. DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber.

[2] Provided by Cargill Inc. (China) and contained corn, corn bran, corn germ meal, spray corn bran, wheat bran, wheat flour, soybean meal, yeast and molasses as the main ingredients.

2.3. Growth measurements, feed intake and diarrhea check

The body weight (BW) and body structures, including withers height and heart girths, were measured on d 1, 14, 28, 42 and 56 (Fig. 1). Starter intakes were recorded before each morning feeding based on the amount offered and refused. Starter samples (Table 1) were collected and analyzed every two weeks for dry matter (DM), crude protein, ether extract, crude ash, neutral detergent fiber (NDF), and acid detergent fiber (ADF) following the methods of AOAC (1995). The starch content of the starter was determined as described by Hall (2009). The MR was collected and analyzed monthly for the CP, fat and lactose analysis using an automated near-infrared milk analyzer (Foss, Hillerød, Denmark) at the Beijing DHI Testing Center (Beijing, China). Diarrhea check was determined by fecal scoring. Scores were recorded after morning milk feeding every day based on a 1 to 4 fluidity scoring system according to the guidelines outlined by Larson et al. (1977). Scores were, 1 = firm, well-formed (not hard); 2 = soft, pudding-like; 3 = runny, pancake batter; and 4 = liquid, splatters, pulpy orange juice. All the fecal scores were collected by one independent trained observer through observing fecal matter on the ground or the tail and hindquarters of the calf. A diarrheic day was defined when the fecal score was >2. Diarrhea frequency was calculated with the following equation: Diarrhea frequency = [(number of calves with diarrhea × days of diarrhea)/(total number of calves × days of trial)] × 100%. Calves with diarrhea were treated according to the protocols established by the farm veterinarian without antibiotics. Instead, they were treated with 30–50 g/d kaolin (mixed with MR, twice a day) for the first 2 d and then observed for 1 d; if diarrhea persisted, the calf would receive kaolin treatment for another 3 d maximum. For some dehydrated calves, 0.9% NaCl solution was administered intravenously until they fully recovered.

2.4. Blood collection and analysis

Blood samples of all calves were collected before the morning feeding on d 28 and 56, and 8 individual samples were randomly selected for analysis. Blood was sampled via jugular venipuncture using vacutainer collection tubes containing no anticoagulant. The blood samples were then centrifuged at 3,500 × *g* for 15 min at 4 °C for serum separation, and then was partitioned into aliquots and stored in 1.5-mL microcentrifuge tubes at −20 °C for later analysis. To investigate the immune status and inflammatory response of dairy calves, serum immunoglobulin (Ig; including IgA, IgG and IgM) concentrations were measured by an automatic biochemical analyzer (Hitachi 7600, Hitachi High-Technologies Corporation, Tokyo, Japan). Besides, the concentrations of serum amyloid A (SAA), haptoglobin (HP), IL-1β, IL-10 in serum were measured using ELISA kits (Beijing Kangjia Hongyuan Biotechnology Co., Ltd., Beijing, China) in an enzyme-immunoassay instrument (Multiskan MK3, ThermoFisher Scientific Co., Ltd., Franklin, USA). Oxidative status biomarkers including MDA, total antioxidant capability (T-AOC), total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px) were analyzed according to the kit manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China).

2.5. Slaughter and sample collection

On the last day of the experimental period (d 56), 12 calves (6 per treatment) were randomly selected and humanely sacrificed 2 h after the morning feeding with a xylazine hydrochloride injection through the jugular catheter, at 0.1–0.15 mL/100 kg of BW (Huamu Animal Health Products Co., Ltd, Jilin, China). Exsanguination was performed once the calf reached a surgical plane of anesthesia, and the abdominal cavity was immediately opened. The esophagus and rectum were first ligated and then each segment was identified and tied off to prevent potential cross contamination. Following this, 15 cm intestinal segments from jejunum and ileum were sampled, respectively. The ileum was defined as 30 cm proximal to the ileo-cecal junction; the distal jejunum was defined as 30 cm proximal to the collateral branch of

the cranial mesenteric artery; and the proximal jejunum was defined as 100 cm distal to the pyloric sphincter (Malmuthuge et al., 2015). Subsequently, the jejunal and ileal tissues were cut into three segments. One segment was fixed in 4% paraformaldehyde (Sigma, USA) for histomorphometric microscopy analysis. The other two segments were cut open and washed with sterile ice-cold PBS buffer (pH 7.2) until the solution was clean and colorless, and then mucosa was scraped gently by sterile glass slides. The mucosa samples were placed into sterile RNAase-free tubes and immediately snap-frozen in liquid nitrogen and transferred to a −80 °C freezer for later gene expression identification and microbial analysis.

2.6. Intestinal morphology analysis

The fixed intestinal segments were removed from 4% paraformaldehyde, and dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, and 100%), cleared with xylene and separately embedded in paraffin blocks in a vertical direction. For each block, 3 to 4 μm thick sections were sectioned by LEICA RM2235 microtome and stained with hematoxylin and eosin according to manufacturer's instructions (Leica Microsystems GmbH, Simi Valley, California, United States). Images were obtained using a light microscopy at 40× magnification (CK×53, Olympus Co., Tokyo, Japan). Five microscopic fields per sample were selected to measure villus height (from the tip of the villi to the villus crypt junction) and crypt depth (the depth of the invagination between adjacent villi) using an image analyzer (Image Pro Plus 6.0, Media Cybernetics, Inc., MD, USA).

2.7. Real-time quantitative PCR analysis

Total RNA of mucosa from the ileum and jejunum were extracted by an RNA extraction kit (Sinogene Biotech co., Ltd., China) according to the protocol. RNA samples were treated with DNase I before cDNA synthesis (Appendix Table 1). Reverse transcription was carried out with 1 μg RNA using a cDNA Synthesis Kit (K1641, Thermo Fisher Scientific, Inc., USA) to synthesize the cDNA. The gene expression of mucosa was determined by quantitative reverse transcription PCR (RT-qPCR), which was performed according to the SYBR Premix Ex Taq II instructions (TaKaRa, Shiga, Japan) on a LightCycler 480II System (Roche Applied Science, Indianapolis, IN, United States). Amplifications were performed in triplicate for each sample. The reaction systems and programs of reverse transcription and RT-qPCR are shown in Appendix Tables 2 to 4. Primers for RT-qPCR (Appendix Table 5) were synthesized by Beijing Tianyi Huiyuan Biological Technology Co., Ltd (Beijing, China). The relative expression of target genes to that of the reference gene (β-actin) was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. DNA extraction, amplicon sequencing and taxonomic identification

Total genomic DNA of the intestinal mucosa was extracted using the modified repeated bead beating and column method, as described by Yu and Morrison (2004). Briefly, approximately 0.2 g of tissue sample were added to bead beating tubes and then subjected to bead beating, followed by column purification using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA USA). The V3 to V4 region of the 16 S rRNA gene was amplified using primers F338 (5′- ACTCCTACGGGAGGCAGCAG -3′) and R806 (5′- GGACTACHVGGGTWTCTAAT -3′) using the following program: 95 °C for 5 min, 20 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, and the final extension step of 72 °C for 7 min and holding at 4 °C. Amplicons were extracted from 2% agarose gels and purified using an EZNA Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. The quality and quantity of purified PCR products were evaluated using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE USA). Paired-end sequencing (2 × 250 bp) was performed on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA USA) for paired-end reads of 300 bp at Biomarker Biotechnology Co., Ltd (Beijing, China).

Sequence data were analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) package (https://qiime2.org) (Bolyen et al., 2019). Quality filtering was performed under specific filtering conditions (Phred score <20; reads <220 bp) to obtain the high-quality clean tags according to QIIME 2. The singletons and chimeric sequences were identified and removed by UCHIM (version 8.1) (Edgar et al., 2011), and the sequences with a similarity level of more than 97% were clustered into operational taxonomic units (OTUs) using USEARCH (version 10; http://drive5.com/uparse/) (Edgar 2013). Taxonomic characterization was denominated according to the SILVA bacterial database (Release 132, http://www.arb-silva.de) (Quast et al., 2013). Alpha diversity indices (Chao 1, Shannon, Simpson and observed species) and Good's coverage were calculated by QIIME 2. For beta diversity, principal coordinate (PCoA) analysis of microbial profiles was conducted using UniFrac distance matrices in R (http://www.rstudio.com). PICURUSt 2 was performed to analyze the differential abundant KEGG pathways (level 3).

2.9. Statistical analysis

Based on Gorka et al. (2011), the PROC POWER and the GLMPOWER procedure in SAS were used to yield a sample size with a power of 0.8 under $P < 0.05$. Statistical analyses were performed using a calf as the experimental unit. The homogeneity of variances and normality of the data were tested first using the UNIVARIATE procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA). The PROC MIXED statement of SAS was used with time as repeated measures for BW, body structural measurements, average daily gain (ADG) and blood parameters. The model included the fixed effects of time, treatment, and time × treatment interaction, and calf within treatment as a random effect. Degrees of freedom were calculated using the Kenward-Roger approximation option of the MIXED procedure. The covariance structures (simple covariance structure, compound symmetry, heterogeneous first-order autoregressive structure, first-order ante-dependence structure, completely general [unstructured]) of the repeated measures within-subject were chosen based on the Akaike information criterion. Data for diarrhea frequency were summarized by period (d 1 to 28, d 29 to 56) and analyzed using the Chi-squares test. The Cramer's V test was used for statistical strength tests of the Chi-square. Morphological parameters and gene expression of the small intestine (jejunum and ileum) were also analyzed using a mixed effect model, but with treatment as the only fixed effect, and animal within treatment as a random effect. A Kruskal–Wallis test was used to analyze indicators of alpha diversity. Differences of $P < 0.05$ were considered significant and $0.05 \leq P < 0.10$ were considered a tendency. Intestinal bacteria were compared using linear discriminant analysis effect size (LEfSe) and significant differences were considered by LDA score > 3.5 and P value < 0.05. Spearman's rank correlations were performed between blood metabolites, gene expression, morphological parameters and the relative abundance of jejunum and ileum mucosa-associated bacterial genera. Significant correlations were defined as $-0.5 < \rho < 0.5$, and $P < 0.05$, where ρ is defined as the Spearman rank-order correlation coefficient.

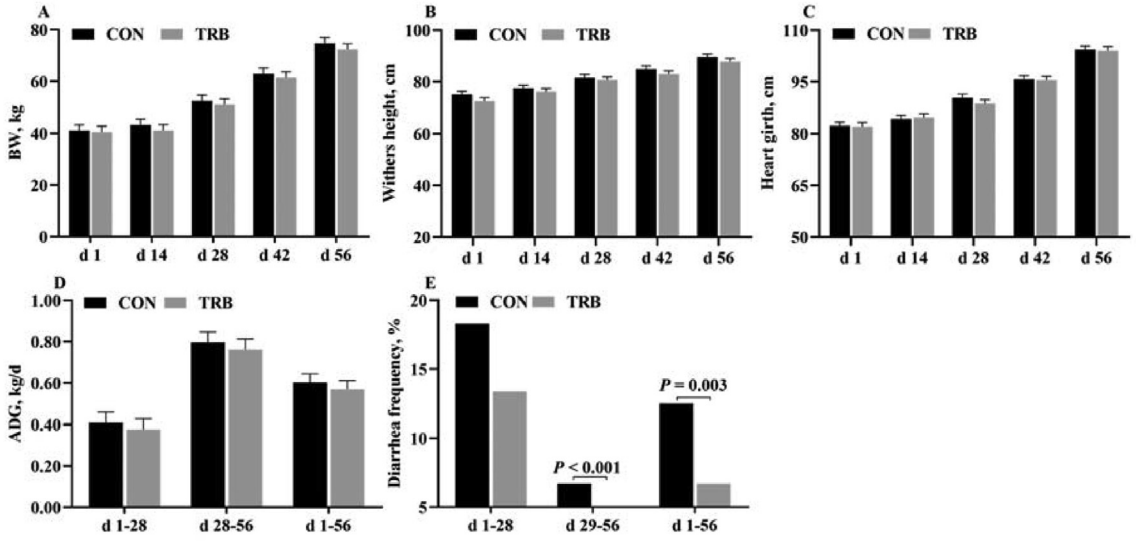

Fig. 2. Effects of tributyrin administration on growth performance and diarrhea of pre-weaning calves (n = 10) (A) Body weight (BW) (mean ± SEM). (B) Withers height (mean ± SEM). (C) Heart girth (mean ± SEM). (D) Average daily gain (ADG) (mean ± SEM). (E) Diarrhea frequency. CON = control, supplemented with no tributyrin; TRB = tributyrin, supplemented with 2 g/L of milk.

Table 2
Effects of tributyrin administration on starter intake of calves.

Starter intake, g/d	Treatment[1]		SEM	*P*-value		
	CON	TRB		Treatment	Time	Treatment × Time
d 3–28	45.0	68.8	13.4	0.18	<0.001	–
d 28–56	624.4	690.0	83.8	0.59	<0.001	–
d 3–56	334.0	379.3	54.0	0.56	<0.001	0.66

[1] CON = milk replacer supplemented with no tributyrin, TRB = milk replacer supplemented with 2 g/L tributyrin.

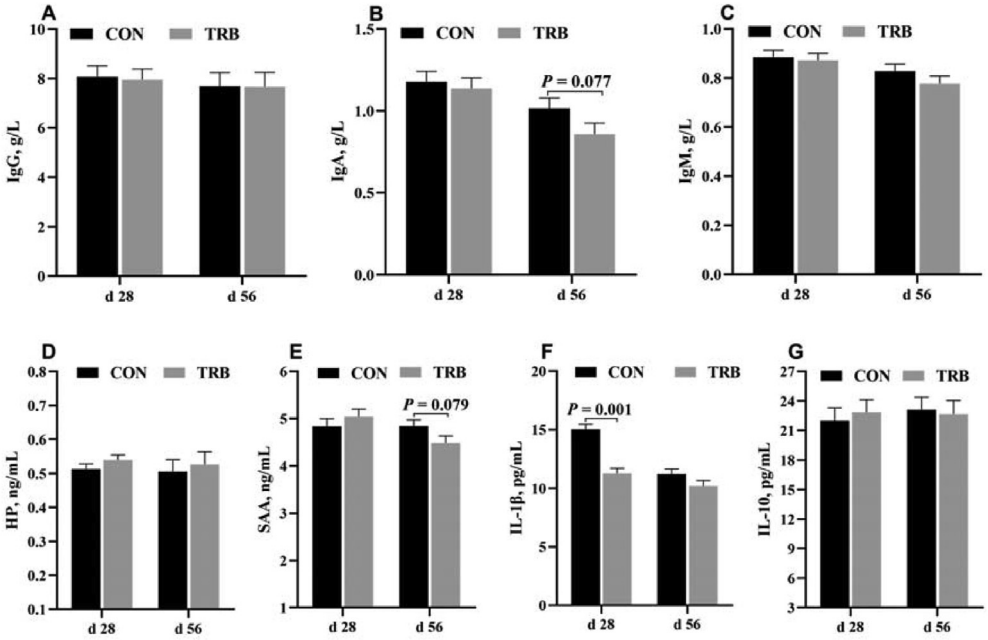

Fig. 3. Effects of tributyrin administration on immune and inflammation-related blood parameters of pre-weaning calves (n = 8) (A to C) Immunoglobulin G (IgG), A (IgA), M (IgM). (D) Haptoglobin (HP). (E) Serum amyloid A (SAA). (F) Interleukin 1β (IL-1β). (G) Interleukin 10 (IL-10). CON = control, supplemented with no tributyrin; TRB = tributyrin, supplemented with 2 g/L of milk.

3. Results

3.1. Growth performance, starter intake and diarrhea frequency

As shown in Fig. 2, tributyrin supplementation had no significant effects on BW, body measurements and ADG. Calves in the TRB group had a significantly lower diarrhea frequency from d 29 to 56 (0 vs. 6.70%, $P < 0.001$) and during the whole period (6.70% vs. 12.5%, $P = 0.003$) compared with calves in the CON group. The starter intake of all calves increased significantly with age ($P < 0.001$), while no significant differences were found in starter intake between treatments from d 3 to 28, d 29 to 56 and the whole period (Table 2).

3.2. Serum immune and anti-oxidant parameters

The concentration of IgA tended to decrease with tributyrin administration on d 56 ($P = 0.077$), while no significant effects were observed on IgG and IgM concentrations (Fig. 3A–C). A treatment × week effect ($P = 0.002$) was observed for IL-1β during the whole period, and tributyrin administration significantly reduced the concentration of IL-1β on d 28 ($P = 0.001$) but no significant differences were found on d 56. The concentration of SAA tended to decrease on d 56 ($P = 0.079$), whereas no significant differences were found in HP and IL-10 (Fig. 3D–G) in TRB compared to the CON group. No significant differences were found in the blood concentrations of serum anti-oxidant parameters, including T-AOC, T-SOD, GSH-Px and MDA between treatments on d 28 or 56 (data not shown).

3.3. Small intestinal morphology

As shown in Fig. 4, tributyrin increased the villus height ($P < 0.001$) and the villus height-to-crypt depth (V/C) ratio ($P = 0.046$) but had no significant effects on crypt depth of the jejunum. In addition, the villus height of the ileum tended to be greater in the TRB group compared to the CON group ($P = 0.074$).

3.4. Intestinal immune and barrier functions

The gut immune and barrier functions in the jejunum and ileum of calves on d 56 were evaluated (Fig. 5). The results showed that administering tributyrin significantly downregulated the gene expression of toll-like receptor 2 (*TLR2*, $P = 0.045$) and tended to downregulate the gene expression of *IL-1β* ($P = 0.088$), but had no significant effect on the gene expression of *IL-10* in the jejunum. In addition, tributyrin administration significantly upregulated the gene expression of claudin-4 ($P = 0.022$), though no significant differences were found in the gene expressions of zonula occludin 1

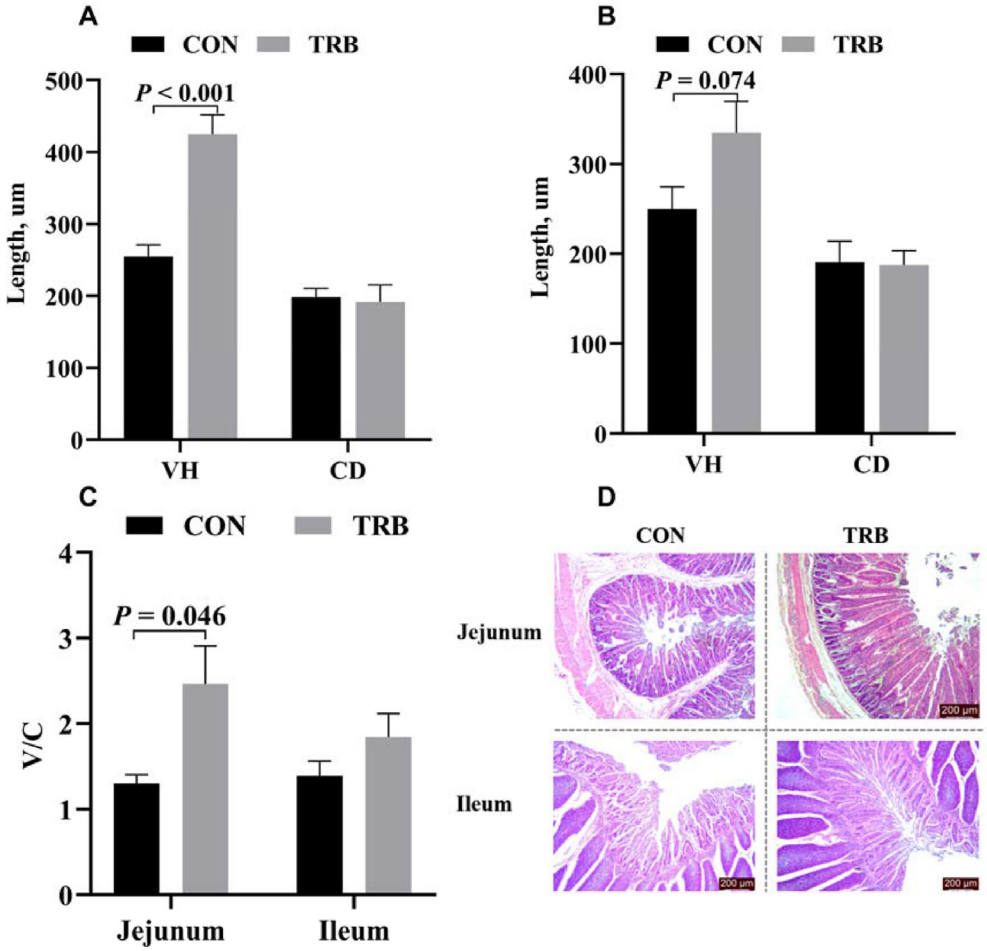

Fig. 4. Effects of tributyrin administration on intestinal morphology of calves on d 56 ($n = 6$). Light microscopy of the villus height and crypt depth of the jejunum (A) and ileum (B) in calves. (C) The ratio of villus height-to-crypt depth (V/C) of the jejunum and ileum. (D) The intestinal morphology of the jejunum and ileum. VH = villus height; CD = crypt depth; CON = control, supplemented with no tributyrin; TRB = tributyrin, supplemented with 2 g/L of milk.

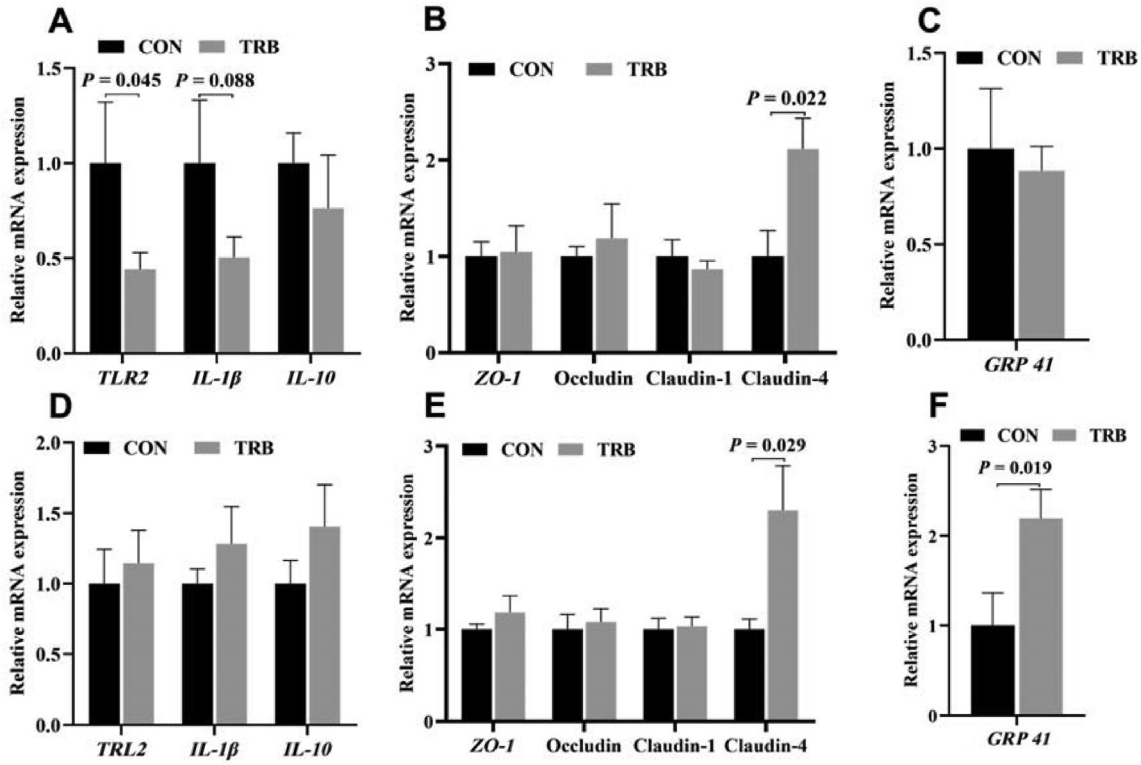

Fig. 5. Effects of tributyrin administration on Toll-like receptor 2 (*TLR2*), gene expressions of tight junctions and G-protein coupled receptor 41 (*GPR 41*) of calves on d 56 ($n = 6$). (A to C) Gene expression in the jejunum; (D to F) Gene expression in the ileum. *ZO-1* = zonula occludin 1; *IL-1β* = interleukin 1β; *IL-10* = interleukin 10. CON = control, supplemented with no tributyrin; TRB = tributyrin, supplemented with 2 g/L of milk.

(*ZO-1*), occludin or claudin-1 in the jejunum. In the ileum, administrating tributyrin had no significant effects on gene expressions including *TLR2*, *IL-1* and *IL-10*. Although no significant effects were observed on the gene expressions of *ZO-1*, occludin or claudin-1 in the ileum, the gene expression of claudin-4 was significantly elevated in the TRB group ($P = 0.029$). The gene expression of G-protein coupled receptor 41 (*GPR 41*) was significantly upregulated in the ileum ($P = 0.019$), yet no significant effect was found in the jejunum.

3.5. Compositional profiles of the intestinal microbiota and taxonomic differences

A total of 758,772 and 710,338 effective sequences were obtained from the jejunal ($n = 6$ per treatment) and ileal ($n = 6$ per treatment) samples, respectively. Based on 97% sequence similarity, 3,777 and 3,706 OTUs were identified in the jejunum and ileum, respectively. In the jejunum, in spite of the 1,845 common OTUs, 203 OTUs were solely isolated in the CON group and 1,729 OTUs presented in the TRB group (Fig. 6A). Moreover, 2,293 common OTUs were identified in ileum, and 520 OTUs were solely isolated in the CON group and 893 OTUs presented in the TRB group (Fig. 6B). The indices of α diversity showed that administering tributyrin significantly increased the Shannon index ($P = 0.045$) and tended to increase the Simpson index ($P = 0.086$), but no significant differences were found in Chao 1 and ACE indexes in the jejunal microbiota (Fig. 6C). Administering tributyrin had no significant effects on the same indices in the ileal microbiota (Fig. 6D). The principal coordinate analysis (PCoA) based on the Unweighted UniFrac distances showed that bacteria tended to be separated between the 2 treatments in the jejunum ($R = 0.235$, $P = 0.075$), whereas a great similarity was found in the ileum (Fig. 7).

Compositional profiles of the small intestinal microbiota were showed in Appendix Fig. 1 and 2. Discriminatory features were observed in the bacterial relative abundance at the phylum and genus levels with an identical threshold that meant relative abundance in one group was more than 0.5%. The dominant bacterial phyla included Bacteroidetes, Firmicutes, and Proteobacteria in both jejunum and ileum. At the genus level in the jejunum, *Prevotella_7*, *Intestinibacter*, and *Escherichia-Shigella* were dominant microbiota in the CON group, whereas *Bacteroidales_S24-7_group*, *Rikenellaceae_RC9_gut_group*, and *Lachnospiraceae_NK4A136_group* were the dominant microbiota in the TRB group. In the ileum, *Escherichia-Shigella*, *Nicotiana_otophora*, and *Turicibacter* were dominant microbiota in the CON group. Similarly, *Bacteroidales_S24-7_group*, *Rikenellaceae_RC9_gut_group* and *Lachnospiraceae_NK4A136_group* were the dominant microbiota in the ileum of the TRB group. LEfSe analysis with LDA values of taxa higher than 3.5 was performed for differential abundance comparison between the CON and TRB group (Fig. 8A and B). No significantly higher abundances of microbiota were found in the jejunum or ileum of calves in the CON group. In the jejunum, calves in the TRB group had significantly higher abundances of Rikenellaceae and Ruminococcaceae families compared with those in the CON group (LDA >3.5, $P < 0.05$), and *Rikenellaceae_RC9_gut_group* was the most abundant genus in the TRB group, followed by *Lachnospiraceae_NK4A136_group* (LDA >3.5, $P < 0.05$). In the ileum, Ruminococcaceae and Lachnospiraceae families exhibited significantly higher abundances in the TRB group (LDA >3.5, $P < 0.05$), and *Bacteroidales_S24_7_group* and *Lachnospiraceae_NK4A136_group* were the top two most abundant genera (LDA >3.5, $P < 0.05$). Moreover, several taxa were more abundant in both the jejunum and ileum of the TRB group, including *Ruminococcus_1*, *Ruminoco-*

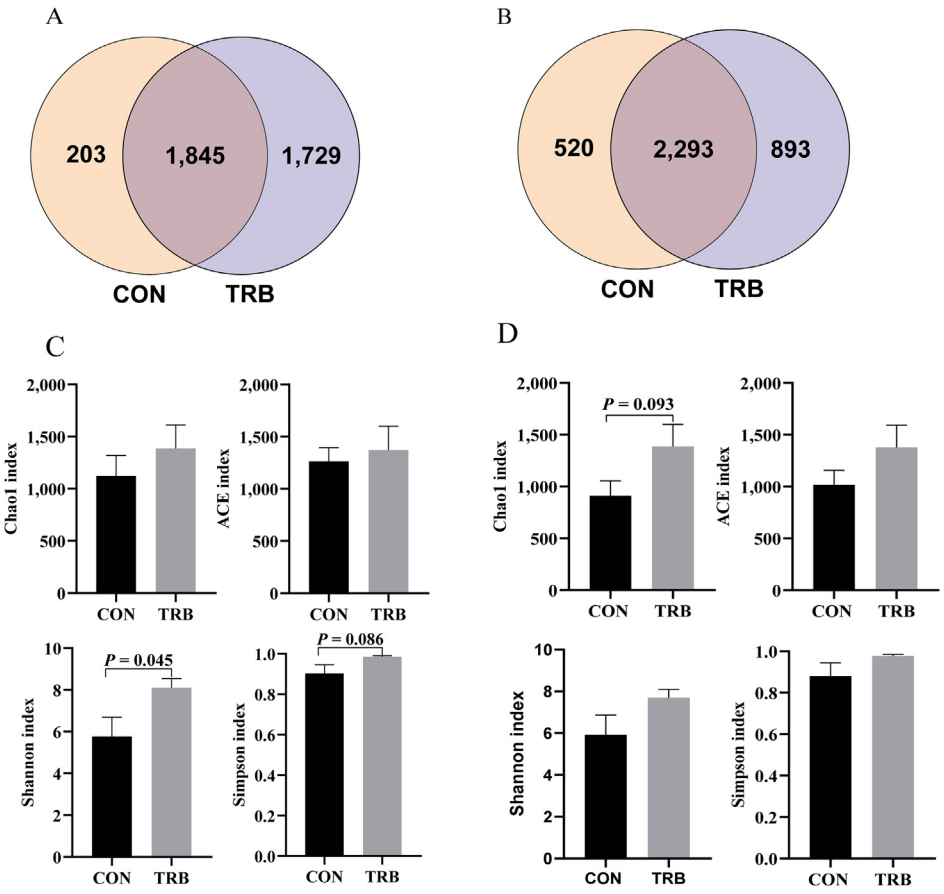

Fig. 6. Effects of tributyrin administration on intestinal microbiota composition of calves on d 56 (n = 6). (A) Venn diagram of jejunum. (B) Venn diagram of ileum. (C) Alpha-diversity in jejunum. (D) Alpha-diversity in ileum. CON = control, supplemented with no tributyrin; TRB = tributyrin, supplemented with 2 g/L of milk.

ccaceae_UCG_013, Ruminococcaceae_UCG_010, Ruminococcaceae_*UCG_005, Lachnospiraceae_NK4A136_group, Alloprevotella* (LDA >3.5, $P < 0.05$).

3.6. Spearman correlation analysis between microbiota, blood parameters and intestinal immune and barrier function associated genes

As shown in Fig. 9A, Ruminococcaceae and Lachnospiraceae had a negative correlation with gene expressions of *TLR2* and *IL-1β* in the jejunum and concentration of blood IL-1β ($P < 0.05$), but a positive correlation with claudin-4 gene expression and villus height ($P < 0.01$). Rikenellaceae had a negative correlation with gene expression of *TLR2* and blood IL-1β ($P < 0.05$), but a strong positive correlation with the villus height of the jejunum ($P < 0.01$). In addition, *Prevotella* was negatively correlated with blood IL-1β concentration ($P < 0.05$), but positively correlated with villus height of the jejunum ($P < 0.001$). The gene expression of *GPR 41* and blood SAA had no significant correlations with any microbiota taxa. Compared with the jejunum, fewer correlations were observed between microbial genera and intestinal immune and barrier function associated genes, blood parameters and morphological parameters in the ileum (Fig. 9B). The gene expressions of *TLR2* and *IL-1β* and the concentration of SAA in blood were not correlated with any genus in the ileum. The significantly enriched genera Ruminococcaceae and Lachnospiraceae in the ileum of the TRB group were negatively correlated with blood IL-1β concentration ($P < 0.05$).

4. Discussion

Previous studies have shown that butyrate supplementation could improve rumen development by increasing the concentration of butyrate in the rumen (Mentschel et al., 2001) and positively affecting the morphological (Gorka et al., 2011; Kato et al., 2011) and metabolic (Naeem et al., 2012; Yan et al., 2014) functions of the ruminal epithelial cells. Besides, Gorka et al. (2014) and Guilloteau et al. (2009) determined that butyrate could also stimulate small intestinal development by increasing the villi length, mucosa thickness, and the activity of enzymes (such as lactase, maltase etc.) and by decreasing apoptosis in calves. These positive effects of butyrate on the GIT may eventually lead to better growth performance of dairy calves. However, studies showed differences in the effects of butyrate on growth performance. Hill et al. (2007) and O'Hara et al. (2018) reported that butyrate increased BW, ADG or structural growth during the pre-weaning period, whereas Slusarczyk et al. (2010) found such an effect mainly after weaning. We did not observe any significant differences in growth performance and starter intake between the treatments in the current study, which is similar to our findings in dairy female calves over the pre-weaning period (Liu et al., 2021). The reasons for incongruence in studies could be attributed to the doses of butyrate applied, duration of supplementation and management of animals (e.g. milk feeding strategies). Perhaps butyrate action during the pre-weaning period stimulated GIT development as we found tributyrin supplementation increased intestinal villi length and the ratio of V/C in the jejunum as well as villi length in the ileum, which

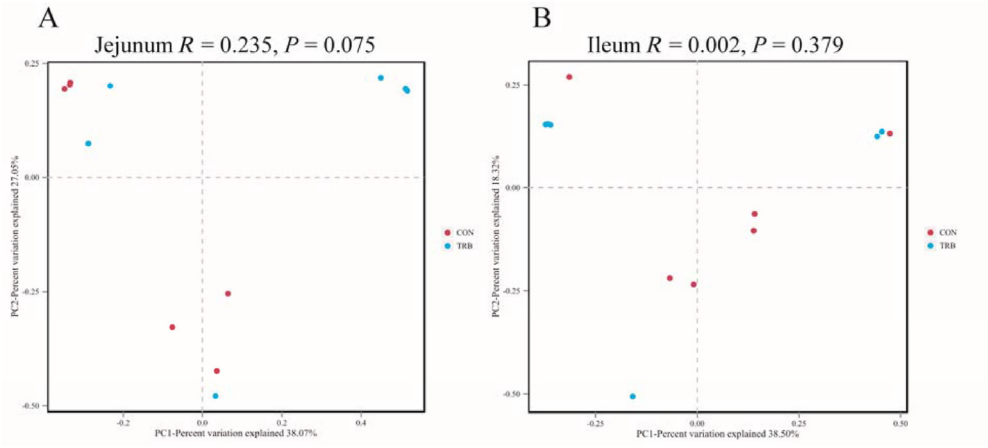

Fig. 7. Effects of tributyrin administration on intestinal microbiota beta-diversity of calves on d 56 (n = 6). Beta-diversity of principal coordinate analysis (PCoA) based on Unweighted UniFrac distances of jejunum (A); beta-diversity of PCoA based on Unweighted Unifrac distances of ileum (B). CON = control, supplemented with no tributyrin; TRB = tributyrin, supplemented with 2 g/L of milk.

is consistent with the findings of Gorka et al. (2014). Therefore, these positive effects of butyrate on GIT development may translate into greater performance in the postweaning period but not the pre-weaning period. As for diarrhea, calves in the TRB group had a reduced diarrhea frequency from d 29 to 56 and the whole period, but it did not show any advantage on reducing the diarrhea frequency from d 1 to 28. Calves are more susceptible to diarrhea during week 2 to 3 after birth which might be suppressed by feeding butyrate within week 1 after birth, as Gorka et al. (2011, 2014) reported reduced fecal scours from d 8 to 14, improved rumen and small intestinal development (d 26) when butyrate was supplemented from d 5. Improved GIT development may help reduce the negative effects of diarrhea on the GIT. In the current study, we observed a numerical decrease in diarrhea frequency (13.4% vs. 18.4%) from d 1 to 28 in calves supplemented with tributyrin, which was inconsistent with the study by Gorka et al. (2011). Perhaps, improved intestinal development due to tributyrin supplementation could not fully ameliorate the negative effects of diarrhea in our study. However, due to the inaccessibility of the small intestine, we could not delineate the effects of tributyrin on intestinal development in the first month of this trial. The positive effects on blood parameters and small intestinal development on d 56 in our study point to carryover effects of tributyrin supplementation from the first week to later in life. Moreover, taking the poor performance in the CON calves and lower IL-1β level in the TRB calves on d 28 into account, tributyrin supplementation, at least partially, reverses the negative effects of diarrhea.

The role of butyrate on anti-inflammation, mucosal immunity, intestinal integrity and barrier functions has been investigated in various mammalian species. In humans and farm animals, butyrate has been shown to inhibit proinflammatory cytokines including IFN-γ, TNF-α, IL-1β, IL-6, and IL-8, and upregulate IL-10 and TGF-β (Bach Knudsen et al., 2018; Byrne et al., 2015). The most studied mechanism underlying the anti-inflammatory action of butyrate is the inhibition of nuclear factor kappa B (NF-κB) (Aguilar et al., 2014; Hudcovic et al., 2012; Lakhdari et al., 2011), which controls the expression of a variety of genes that encode for proinflammatory cytokines and enzymes, adhesion molecules, growth factors, acute-phase proteins, and immune receptors (Elce et al., 2017; Jobin and Sartor 2000). In the present study, the gene expression of *IL-1β* was suppressed in the jejunum, while its concentration in the blood was reduced by tributyrin supplementation on d 28 though only a numerical reduction was found on d 56. Besides, the concentration of SAA in blood decreased with tributyrin supplementation. SAA, one of the most important bovine APPs, can be used as a biomarker

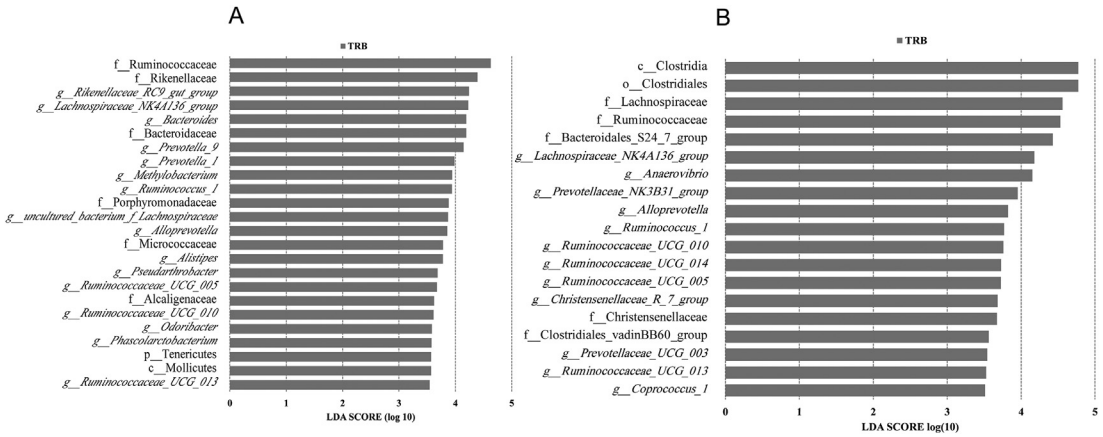

Fig. 8. Differential intestinal bacteria of jejunum (A) and ileum (B) between CON and TRB. Significant differences were tested by linear discriminant analysis effect size (LEfSe) analysis with linear discriminant analysis (LDA) score > 3.5 and P-value < 0.05. CON = control, supplemented with no tributyrin; TRB =tributyrin, supplemented with 2 g/L of milk.

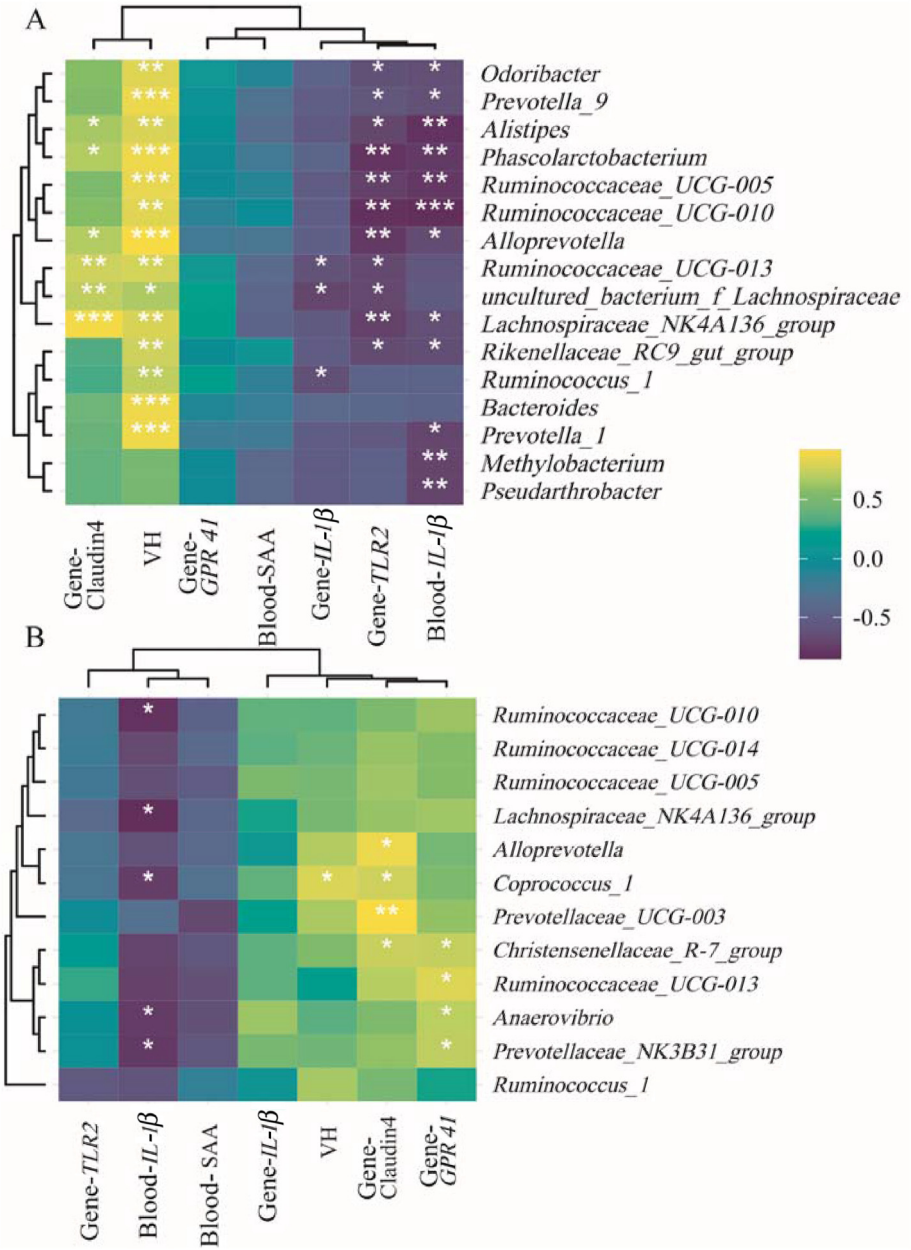

Fig. 9. Spearman correlation analysis between microbiota and intestinal immune and barrier function associated genes, blood parameters and morphological parameters in the jejunum (A) and ileum (B). Significant different genes (Toll-like receptor 2 [*TLR2*], interleukin 1β [*IL-1β*], claudin-4, G-protein coupled receptor 41 [*GPR 41*]), blood parameters (IL-1β, serum amyloid A [SAA]) and morphological parameters (villus height, VH) were analyzed with significant different microbial genera in the jejunum and ileum. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

responding to infection, inflammation and trauma (Eckersall and Bell 2010). It increases in blood during the acute-phase reaction but is present in low levels in healthy animals (Gånheim et al., 2003; Murata et al., 2004). These results together indicated a positive effect of tributyrin supplementation on alleviating inflammation in dairy calves, which is similar to those found in piglets and broilers (Bortoluzzi et al., 2017; Huang et al., 2015). We also found that the gene expression of *TLR-2* was downregulated. *TLR-2* plays an important role in activation of NF-κB through TLR—NF-κB signaling pathways (Kawai and Akira 2007), thus the reduced gene expression of *TLR-2* further confirmed the anti-inflammatory action of butyrate in dairy calves. *GPR 41*, one of the most important receptors of SCFAs, was elevated in the ileum of calves receiving tributyrin in this study. Consistently, the relative abundance of SCFA-producing microbiota increased in calves supplemented with tributyrin, which might have enhanced SCFA production and eventually contributed to the upregulation of *GPR 41*. *GPR 41* accompanied with *GPR 43* could activate extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase signaling pathways in epithelial cells to induce rapid production of chemokines and cytokines during immune responses, and eventually mediate protective immunity and tissue inflammation (Kim et al., 2013).

Butyrate is also well known for maintaining intestinal integrity and barrier function. Tight junction proteins such as occludin and claudins can bind to zonula occludens proteins and build up the primary barrier to pathogens (Hotamisligil and Bernlohr 2015). At the cellular level, Wang et al. (2012) documented that butyrate

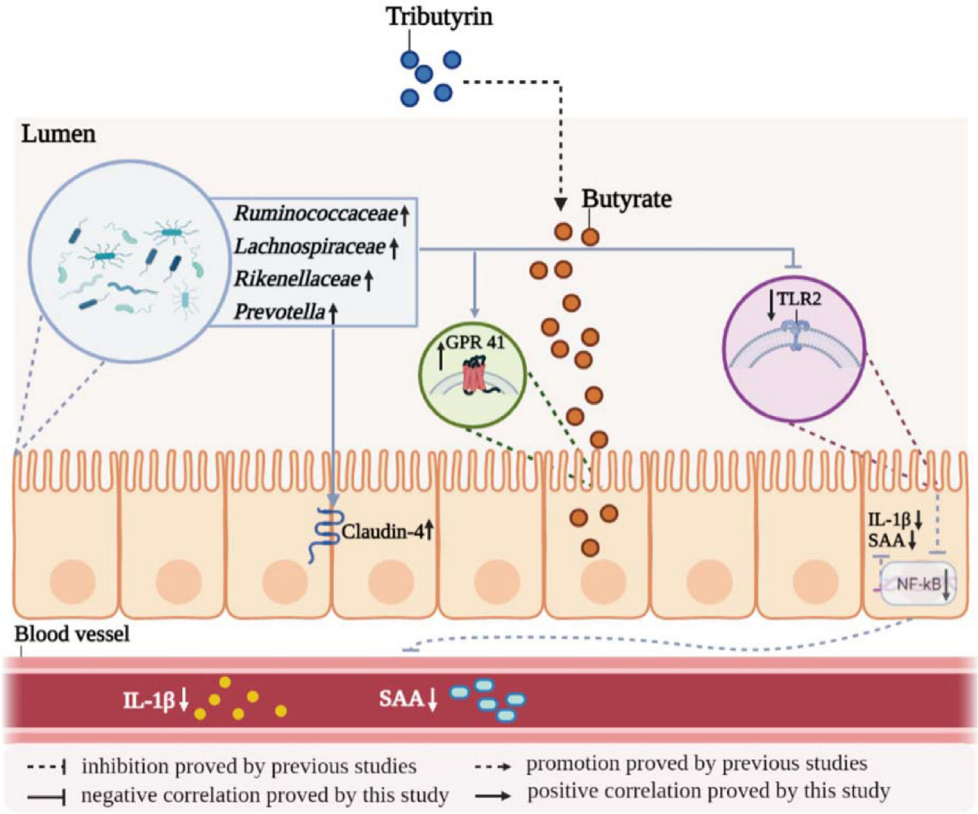

Fig. 10. Graphical summary of the effect of tributyrin administration on the intestinal development and health of pre-weaned dairy calves. Tributyrin oral delivery stimulated SCFA-producing bacteria colonization, enhanced intestinal barrier functions, suppressed inflammatory response and consequently improved intestinal development and health of pre-weaned dairy calves. TLR2 = toll-like receptor 2, IL-1β = interleukin 1β, GPR 41 = G-protein coupled receptor 41, SAA = serum amyloid A. nuclear factor kappa B = NF-κB.

could enhance intestinal epithelial barrier function via up-regulation of tight junction protein claudin-1 transcription. When added to feed, butyrate could also improve tight protein transcription in poultry (Song et al., 2017). Similarly, we also found upregulated gene expression of claudin-4 both in the jejunum and ileum of the TRB group, which suggested that the intestinal physical barrier that protects intestines from pathogenic infection was enhanced in dairy calves. The positive effects of butyrate on intestinal integrity and barrier function may eventually lead to lower diarrhea frequency of calves supplemented with tributyrin in the present study and other studies (Gorka et al., 2011; Hill et al., 2007).

The role of gut microbiota in the development and health of the GIT across animal species has been widely recognized. Gut microbiota manipulation during early life, a key factor that influences gut health (Malmuthuge and Guan 2017), is one approach to improve calf gut health. Butyrate and its derivatives have been widely used as feed additives to protect the animal's gut health. However, limited research has been conducted on the microbial mechanisms that contribute to the protective effects of butyrate on the gut. Consistent with the present study, previous research (Bernad-Roche et al., 2021; Wu et al., 2018) showed that microbial α diversity was similar, while β diversity was different in both pigs and poultry between supplemented with or without butyrate. These results indicate a potential effect of butyrate supplementation on the structure of intestinal microbiota. Like in our study, Bernad-Roche et al. (2021) and Wu et al. (2018) reported that Lachnospiraceae was increased with butyrate supplementation. Meanwhile, we also found that the abundance of Ruminococcaceae was significantly higher in the TRB group, and both of them had a strong negative correlation with inflammation-related gene expression but a positive correlation with barrier function-related gene expression and villus height. The Lachnospiraceae and Ruminococcaceae are two of the most abundant families in the order Clostridiales and share a common role in degrading complex polysaccharides to short-chain fatty acids that can be used as an energy source by the host (Biddle et al., 2013), which contributes to the development of intestinal epithelial cells and morphological structure. Additionally, Lachnospiraceae and Ruminococcaceae have been positively associated with healthy controls and characterized as anti-inflammatory commensal bacteria. As such, Lachnospiraceae and Ruminococcaceae were shown to be depleted in inflammatory bowel disease (Frank et al., 2007) and Crohn's disease (Fujimoto et al., 2013), respectively. The anti-inflammatory mechanism of these 2 bacteria was highly related to their properties of producing SCFA butyrate. Furthermore, *Prevotella* was also enhanced in calves supplemented with tributyrin in this study and negatively correlated with gene expression of *IL-1β* but positively correlated with gene expression of claudin-4 and villus height of the jejunum. *Prevotella* has been known for fermenting complex dietary polysaccharides to produce energy for intestinal development (Mach et al., 2015). Besides, *Prevotella* could produce acetate, which is further used by other microbial species to produce butyrate and thus contributes to the maturation of mucosal immunity and helps to maintain the anti/pro-inflammatory balance (Ratajczak et al., 2019). The results in the present study further highlighted the positive effects on intestinal development and health of the above three bacteria in dairy calves.

Rikenellaceae, Porphyromonadaceae and Christensenellaceae were also of higher abundance in the jejunum and ileum of calves in the TRB group. However, no association was observed between them and intestinal immune and barrier function associated genes or blood parameters. Rikenellaceae, a newly established family in the order Bacteroidales, is placed near Porphyromonadaceae, and has been found to be positively correlated with the production of acetate and butyrate (Wang et al., 2020). Both Rikenellaceae and Porphyromonadaceae were reported to be enriched in healthy groups but suppressed in those with greater inflammation (Jiang et al., 2015). Moreover, Christensenellaceae has recently been found to be highly correlated with Rikenellaceae and associated with the metabolism of several sugars and acetate and butyrate production (Morotomi et al., 2012; Oki et al., 2016). In a meta-analysis of inflammatory bowel disease that included over 3000 individuals, Morotomi et al. (2012) reported Christensenellaceae as one of five taxa considered the signature of a healthy gut. Therefore, the higher abundance of Rikenellaceae, Porphyromonadaceae and Christensenellaceae in the TRB group may indicate their protective effects on the small intestine in dairy calves, whereas the mechanism needs to be further studied.

In this study, we applied 16 S rRNA gene amplicon sequencing and molecular biology techniques and demonstrated the positive effects of supplementing exogenous butyrate in MR on intestinal development and health of dairy calves (Fig. 10). However, the number of calves (6 calves per group) used might not have been adequate to appropriately test the effects of tributyrin on intestinal development. Studies in larger cohorts, possibly at different physiological stages (pre- or post-weaning), via advanced techniques such as shotgun metagenomics combined with metabolomics, will further enhance our understanding and provide insight into the mechanisms underlying the relationship between gut microbiota and intestinal immune and barrier function associated genes in dairy cattle. Moreover, we found out that tributyrin supplementation had a greater effect on the development and health of the jejunum compared to the ileum. Similarly, Gorka et al. (2014) described more positive effects (improved morphology and enzyme activities) of butyrate supplementation on the jejunum rather than the ileum. The discrepancies in morphology, physiology and microbial composition between the jejunum and ileum may account for this (Malmuthuge and Guan 2017; Steele et al., 2016).

5. Conclusions

Tributyrin oral delivery could reduce the concentration of IL-β and SAA in blood, and downregulate the gene expression of *TLR2* and *IL-1β* but upregulate the gene expression of claudin-4 and *GPR 41* in pre-weaned dairy calves. In addition, administering tributyrin improved small intestinal morphological parameters. Moreover, supplementing tributyrin significantly increased the abundance of SCFA-producing bacteria including Ruminococcaceae, Lachnospiraceae, Prevotella and Rikenellaceae, which were negatively associated with inflammatory gene expression but positively associated with intestinal barrier gene and morphological parameters. Therefore, tributyrin supplementation stimulated SCFA-producing bacteria colonization, enhanced intestinal barrier function, suppressed inflammatory response and consequently improved intestinal development and health of pre-weaned dairy calves.

Author contributions

Zhijun Cao: Conceptualization, Supervision, Funding acquisition; **Shuai Liu** and **Junda Wu**: Investigation, Data curation, Formal analysis, Writing- Original draft preparation; **Zhaohai Wu**, **Jinghui Li**, **Gibson Maswayi Alugongo**, **Jianxin Xiao**, **Zhiyuan He** and **Yulin Ma**: Investigation, Writing- Reviewing and Editing, Visualization; Shengli Li: Writing- Reviewing and Editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

We want to thank Jie Zhou, Sihan A, Kebi Hao, Na Lu for their assistance in sample collection. We are grateful to the staff of Dairy Education and Research Centre (Shunyi, Beijing, China) for their assistance with the trial. The work was supported by the National Key Research and Development Program of China, China (2021YFF1000703-03) and the Research Innovation Fund for Graduate Students of CAU, China (2021TC116) and the grant from Guangdong VTR Biotechnology Co., Ltd (2017DR05, Guangdong, China).

Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.06.004.

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