



## Original Research Article

# Sodium butyrate promotes gastrointestinal development of preweaning bull calves via inhibiting inflammation, balancing nutrient metabolism, and optimizing microbial community functions



Huiyue Zhong<sup>a</sup>, Wenjing Yu<sup>b</sup>, Min Wang<sup>c</sup>, Bo Lin<sup>b</sup>, Xuezhao Sun<sup>d, e</sup>, Nan Zheng<sup>a</sup>, Jiaqi Wang<sup>a, \*</sup>, Shengguo Zhao<sup>a, \*</sup>

<sup>a</sup> State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

<sup>b</sup> Department of Animal Science and Technology, Guangxi University, Nanning, 530005, China

<sup>c</sup> Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, 410125, China

<sup>d</sup> Jilin Inter-regional Cooperation Centre for the Scientific and Technological Innovation of Ruminant Precision Nutrition and Smart and Ecological Farming, Jilin Agricultural Science and Technology University, Jilin, 132109, China

<sup>e</sup> Grasslands Research Centre, AgResearch Limited, Palmerston North, New Zealand

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## ABSTRACT

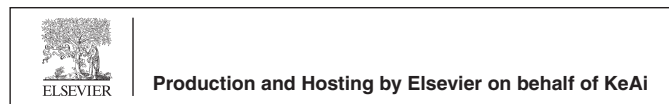
Butyrate promotes the growth and gastrointestinal development of calves. But, the mechanisms behind its effects on signaling pathways of the gastrointestinal tract and rumen microbiome is unclear. This study aimed to reveal transcriptomic pathways of gastrointestinal epithelium and microbial community in response to butyrate supplementation in calves fed a high fiber starter. Fourteen Holstein bull calves ( $39.9 \pm 3.7$  kg, 14 d of age) were assigned to 2 groups (sodium butyrate group, SB; control group, Ctrl). The SB group received 0.5% SB supplementation. At d 51, the calves were slaughtered to obtain samples for analysis of the transcriptome of the rumen and jejunum epithelium as well as ruminal microbial metagenome. Sodium butyrate supplementation resulted in a higher performance in average daily gain and development of jejunum and rumen papillae. In both the rumen and jejunum epithelium, SB down-regulated pathways related to inflammation including NF- $\kappa$ B (*PPKCB*, *CXCL8*, *CXCL12*), interleukin-17 (*IL17A*, *IL17B*, *MMP9*), and chemokine (*CXCL12*, *CCL4*, *CCL8*) and up-regulated immune pathways including the intestinal immune network for immunoglobulin A (IgA) production (*CD28*). Meanwhile, in the jejunum epithelium, SB regulated pathways related to nutritional metabolism including nitrogen metabolism (*CA1*, *CA2*, *CA3*), synthesis and degradation of ketone bodies (*HMGCS2*, *BDH1*, *LOC100295719*), fat digestion and absorption (*PLA2G2F*, *APOA1*, *APOA4*), and the PPAR signaling pathway (*FABP4*, *FABP6*, *CYP4A11*). The metagenome showed that SB greatly increased the relative abundance of *Bacillus subtilis* and *Eubacterium limosum*, activated ruminal microbial carbohydrate metabolism pathways and increased the abundance of carbohydrate hydrolysis enzymes. In conclusion, butyrate exhibited promoting effects on growth and gastrointestinal development by inhibiting inflammation, enhancing immunity and energy harvesting, and activating microbial carbohydrate metabolism. These findings provide new insights into the potential mechanisms behind the beneficial effects of butyrate in calf nutrition.

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\* Corresponding authors.

E-mail addresses: [jiaqiawang@vip.163.com](mailto:jiaqiawang@vip.163.com) (J. Wang), [zhaoshengguo1984@163.com](mailto:zhaoshengguo1984@163.com) (S. Zhao).

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## 1. Introduction

The development of the gastrointestinal tract (GIT) plays an important role in the growth of calves (Steele et al., 2016). The growth performance and GIT development while calves are still developing have a long-term influence on the production performance and health of adult cattle (Khan et al., 2016; Meale et al., 2017; Soberon et al., 2012). Thus, it is important to ensure that

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the GIT of a calf is well developed and healthy in order to relieve the stress of weaning.

The dietary supplementation of sodium butyrate (SB) is one of the most efficient approaches to the improvement of GIT development. Butyrate in the diet can increase feed intake and body weight of calves (Nazari et al., 2012), stimulate the development of rumen papillae, promote the proliferation of epithelial cells (McCurdy et al., 2019; Mentschel et al., 2001), and enhance rumen fermentation (McCurdy et al., 2019). The effects of butyrate on the biological function are via either affecting short chain fatty acid-sensing G-protein-coupled receptors (Milligan et al., 2017) or inhibiting the activity of histone deacetylase (Krautkramer et al., 2016). Supplementary SB affects not only rumen development but also gut cell proliferation and development by providing energy (Bergman, 1990; Górká et al., 2014, 2018a). However, most studies supplemented SB in the calf diet with a low level of fiber. Effects of butyrate on the growth of calves especially with a high fiber starter and its regulation target are still unclear. Therefore, we hypothesized that dietary butyrate supplementation can promote animal growth and development by enhancing some specific functional pathways and by influencing rumen microbial community under a high fiber starter. The aim of the study was to investigate which processes are affected by butyrate in the GIT to promote the growth and development of preweaning calves and how butyrate influences rumen microbial functions.

## 2. Materials and methods

### 2.1. Animal ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of the Institute of Animal Science, Guangxi University (No. GXU-2019-214).

### 2.2. Animals, treatments and feeding

A total of 14 healthy Holstein bull calves ( $39.86 \pm 3.69$  kg, 14 d of age) were randomly assigned to 2 groups. At the beginning of the experiment, all calves were commenced on the starter pellets. Calves in one group were fed the starter with a high content of fiber (NDF of 30.9% to 31.1% in the starter) and treated as the control group (Ctrl), while calves in the other group were fed the same starter but supplemented with 0.5% sodium butyrate (SB; DM basis) and referred to as the SB group (Table 1). All calves were individually raised and fed with the same amount of milk powder (Beijing Precision Animal Nutrition Research Center, China) twice a day (08:00, 16:00). Each calf was initially provided with 8 L of milk daily with the ratio of milk powder to water at 1:9. The amount of daily milk provided to the calves was gradually decreased by 2 L fortnightly until it reached 4 L per day. This amount was maintained until the end of the experiment. All calves had free access to the starter and water.

Before morning feeding on d 0, 14, 28 and 42, the body weight of the calf was measured for the calculation of average daily gain (ADG). Body size traits including body height, body length, body oblique length, chest circumference, and cannon circumference were also recorded. Daily dry matter intake (DMI) was individually recorded. Before morning feeding on d 14, 28, and 42, the shape of feces was observed and scored based on the 4-score criteria listed in Table S1 (Magalhaes et al., 2008). The experiment lasted for 51 d. All calves were slaughtered by electric shock for rumen content and gastrointestinal tissue collection in the morning of d 51.

**Table 1**

The ingredients and nutritional content of experimental starter diets (DM basis, %).

Item	Treatment	
	Control (Ctrl)	Sodium butyrate (SB)
<b>Ingredients</b>		
Corn	19.6	19.6
Peanut vine	37.0	36.5
Soybean meal	33.0	33.0
Corn gluten meal	5.0	5.0
Limestone powder	0.8	0.8
Soybean oil	1.2	1.2
CaHPO <sub>4</sub>	1.8	1.8
NaCl	0.6	0.6
Sodium butyrate	0.0	0.5
Premix <sup>1</sup>	1.0	1.0
<b>Nutrient contents</b>		
DM	87.6	87.6
Metabolizable energy, MJ/kg <sup>2</sup>	14.6	14.6
CP	20.5	20.5
Starch	18.8	18.7
NDF	1.1	0.9
ADF	9.6	9.5
OM	91.9	91.9
Ash	8.1	8.1

ADF = acid detergent fiber; CP = crude protein; NDF = neutral detergent fiber; OM = organic matter.

<sup>1</sup> The premix supplemented to 1 kg of diet (DM basis) contained: 0.25 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.08 g CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.2 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.15 g ZnSO<sub>4</sub>·2H<sub>2</sub>O, 0.5 mg NaSeO<sub>3</sub>, 1 mg KI, 2 mg CoCl<sub>2</sub>·6H<sub>2</sub>O, 5,000 IU vitamin A, 3,000 IU vitamin D<sub>3</sub>, 50 IU vitamin E.

<sup>2</sup> The metabolic energy was calculated according to NRC (2001).

### 2.3. Sample collection

The starter diet was collected weekly for the determination of nutrient composition. After slaughter, the carcass weight was recorded. The digesta from the rumen was filtered through 4 layers of gauze for pH measurement using a pH meter (SG2, Mettler Toledo, Shanghai, China), and aliquots stored in liquid nitrogen for the analysis of rumen fermentation parameters and microbial metagenomic sequencing. The rumen, reticulum, omasum, and abomasum were weighed. The rumen volume was measured by the method of water infusion (Tummler et al., 2020). The ventral cranial sac epithelium of the rumen without the muscle layer (5 cm × 5 cm) was taken, rinsed in physiological saline until clear, and then stored in 4% paraformaldehyde (PFA) solution for morphological observations and in liquid nitrogen for transcriptomic analysis.

The weight and length of the gut including the jejunum, ileum, colon, duodenum, cecum, and rectum were measured. The epithelial tissues of the jejunum and ileum (1 cm × 1 cm) were taken and stored in 4% PFA solution for morphological observations and in liquid nitrogen for transcriptomic sequencing.

### 2.4. Rumen epithelium morphometry and rumen fermentation

The rumen papillae ( $n = 40$  for each sample) preserved in 4% PFA were cut randomly along the basal layer by a pair of scissors. The length and width of rumen papillae were measured using an electron microscope (SN-0745, SangNond, Shenzhen, China), and the surface area of rumen papillae was calculated as described by Zitnan et al. (2005).

The short chain fatty acid (SCFA) concentrations of rumen fluid were measured using gas chromatography (7890 A, Agilent, Wilmington, DE, USA) equipped with a flame ionization detector using a DB-FFAP column (30 mm × 320 μm × 0.5 μm) as described previously (Mohammed et al., 2004), and NH<sub>3</sub>-N concentration was

detected using the phenol-hypochlorite method (Broderick and Kang, 1980).

### 2.5. Epithelium transcriptomic sequencing and analysis

The total RNA of the rumen and jejunum epithelium was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of total RNA was detected using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). The mRNA sequencing library was constructed by the MGIEasy RNA agent kit (MGI, Shenzhen, China). The mRNA sequencing was performed by MGISEQ-2000 (2 × 150 bp) (MGI, Shenzhen, China).

Raw reads containing adaptors with unknown N base greater than 10% and the low-quality reads (bases with a value less than 15 accounted for more than 50% of the total bases) were removed using SOAPnuke v1.5.6 (Chen et al., 2018) to get clean reads. Clean reads were aligned to the *Bos taurus* reference genome (NCBI No. of UMD3.1) with HISAT v0.1.0-beta (Kim et al., 2015) and Bowtie2 v2.2.5 (Langmead and Salzberg, 2012). The expression levels of genes and transcripts were calculated with RSEM v1.2.12 (Li and Dewey, 2011) expressed as fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed genes (DEG) were detected using DEGseq2 (Love et al., 2014), and only those with fold change  $\geq 2$  and adjusted *P*-value  $\leq 0.05$  were identified as DEG. Differently expressed genes were classified into biological pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation. The enrichment of pathways was estimated using the phyper in R (R core team 2020, Vienna, Austria), and only pathways with FDR  $\leq 0.01$  were considered significant enrichment pathways.

### 2.6. Verification of changed mRNA by quantitative PCR (qPCR)

The differentially expressed genes identified from the transcriptomic analysis were confirmed by SYBR Green-based real-time qPCR assays. The analysis was performed on a Real-Time PCR System (Lightcycle K, IOER, Hangzhou, China) using the method  $2^{-\Delta\Delta C_t}$  method with the reference gene of beta-actin. The primers for all genes are listed in Table S2. The cDNA was synthesized with the Prime Script II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) using epithelium RNA.

The qPCR reaction system (20  $\mu$ L) contained 10  $\mu$ L SYBR Premix Ex Tag (2 ×) (Takara, Dalian, China), 0.8  $\mu$ L forward primer, 0.8  $\mu$ L reverse primer, 1  $\mu$ L DNA template (cDNA), and 7.4  $\mu$ L ddH<sub>2</sub>O. The settings of two-step qPCR amplifying conditions were as follows: (1) amplification curve: 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 20 s, with a single point detection signal at 72 °C, (2) melting curve: 65 °C for 15 s with continuous fluorescence detection.

### 2.7. Rumen microbial metagenomic sequencing and analysis

Rumen digesta samples from 5 calves in each group were randomly selected for metagenomic sequencing. The total DNA of rumen microbes was extracted through the CTAB-based buffer plus bead beating as described by Minas et al. (2011). The concentration and quality of DNA were examined using Nanodrop One (Thermo, Waltham, MA, USA). The sequencing library was generated using MGIEasy Fast FS DNA Library Prep Set (MGI, Shenzhen, China). The metagenomic sequencing was performed using DNBSEQ-T1 (2 × 150 bp) (MGI, Shenzhen, China).

Reads that contained more than 20% bases with a mass value less than 20, or an adaptor, or more than 5% none base, were abandoned using SOAPnuke (v1.5.6) (Chen et al., 2018). To reduce interferences from the host, reads mapped to bovine genome

UMD3.1.69 (>90% identity) based on Bowtie2 (v2.2.5) (Langmead and Salzberg, 2012) were also removed. The clean data were de novo assembled using MEGAHIT (Li et al., 2015). Open reading frames were predicted using MetaGeneMark (v2.10) (Zhu et al., 2010). The merged predicted genes from all samples were clustered (>95% identity and >90% sequence length) by CD-Hit (4.6.6) (Li and Godzik, 2006) to get a nonredundant gene set. Based on the results of aligning using Bowtie2, the gene abundance was estimated using Salmon (v0.9.1) (Patro et al., 2017). Gene functional annotations were obtained by aligning to Swiss-Port, KEGG, eggNOG, NR, GO, CAZy databases using Diamond (v0.8.23.85) (Buchfink et al., 2014). The taxonomy was obtained by aligning to the NR database and analyzing using the lowest common ancestor (LCA) algorithm (Huson et al., 2007). The principal component analysis and alpha diversity were performed by the vegan package in R (R core team 2020, Vienna, Austria). The statistical differences between sample groups were analyzed by the Wilcoxon rank sum test, and the enrichment analysis of differentially expressed genes was performed with the phyper in R package (R core team 2020, Vienna, Austria).

### 2.8. Statistical analysis

The data on calf growth phenotype, gastrointestinal development, and qPCR were analyzed using the GLM model in SAS 9.4 (SAS Institute, Cary, NC, USA), with treatment and age as fixed effects. The best fecal score percentage was analyzed using the Chi-Square test in SAS 9.4. *P* < 0.05 was considered a significant difference.

## 3. Results

### 3.1. Calf growth and gut health

Compared with the Ctrl group, the body weight, carcass weight, DMI and ADG were all improved greatly (*P* < 0.05) in the SB group by 5%, 13%, 16% and 3%, respectively (Table 2). As to body size traits, SB supplementation promoted body height and chest circumference (*P* < 0.05), but not body length, body oblique length, and cannon circumference (*P* > 0.05). The percentage of the best fecal score represented the percentage of score 1 in all scores of 1 to 4. When calves were aged from d 28 to 56, SB increased the rate of the best fecal score (*P* < 0.05), especially at the age of 28 d (*P* < 0.05) (Table S3). Nevertheless, this significant effect was not observed at the age of 42 d or 56 d (*P* > 0.05).

### 3.2. Rumen fermentation and epithelium morphology

The supplementation of SB resulted in greater length and surface area of rumen papillae by 28% and 43% respectively (*P* < 0.05) but had no significant influence on the width of papillae (*P* > 0.05) (Fig. 1). The supplementation of SB also increased butyrate concentration by nearly 3-fold (4.21 vs. 12.22 mmol/L; *P* < 0.01), but did not significantly affect ruminal pH value and the concentrations of NH<sub>3</sub>-N, total SCFA, acetate, and propionate (*P* > 0.05; Table 3).

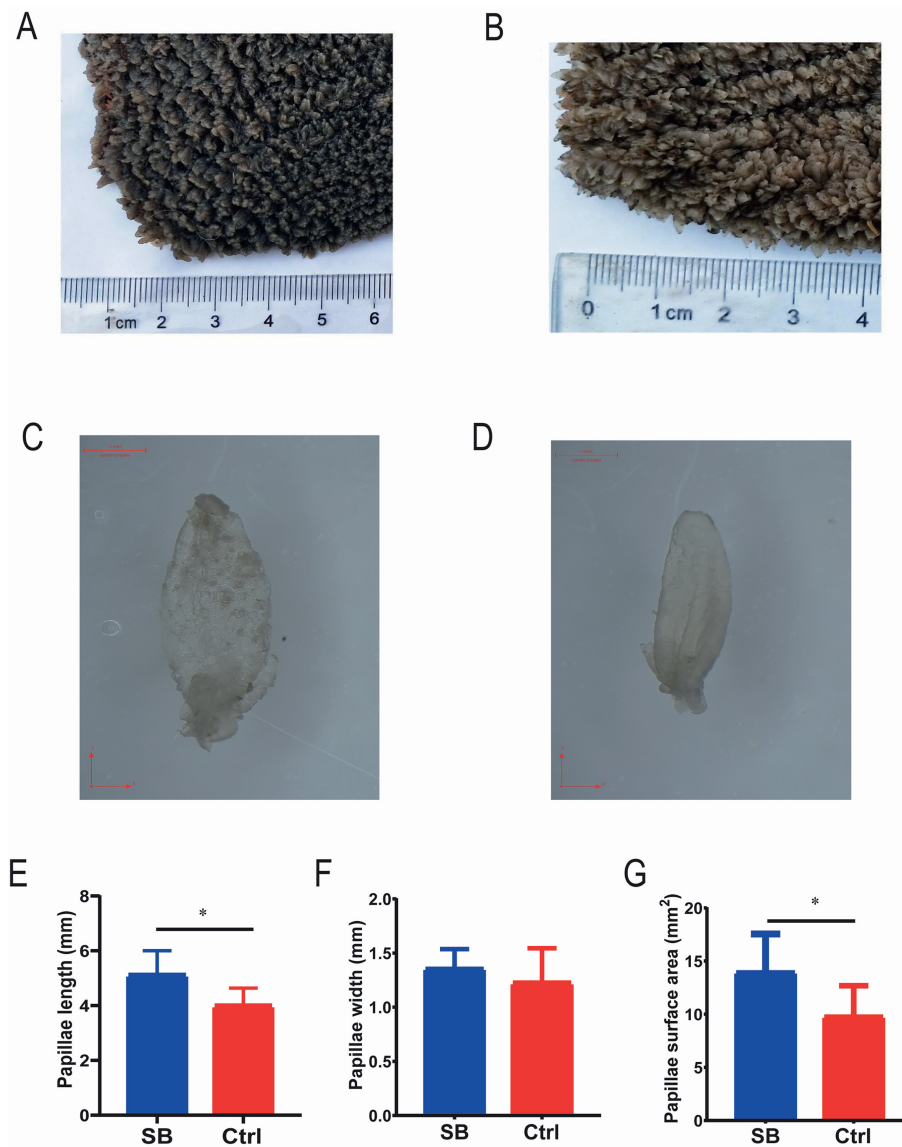
### 3.3. Weight and length of the gastrointestinal tract

During the development of the GIT, SB enhanced the weight of the rumen-reticulum and jejunum by 34% and 25%, respectively (*P* < 0.05; Table 4). With regard to the length of the gut, SB only had a positive effect on the jejunum (*P* < 0.05).

**Table 2**  
Effect of dietary sodium butyrate supplementation on the growth of preweaning bull calves.

Item	Treatment		SEM	P-value
	Control (Ctrl)	Sodium butyrate (SB)		
DMI, g/d	465	541	42.4	<0.01
Body weight, kg	48.5	50.7	1.23	0.02
Carcass weight, kg	30.5	34.5	0.97	0.03
ADG, kg/d	0.40	0.52	0.022	<0.01
Body size, cm				
Body height	79.5	81.2	0.53	0.04
Body length	66.0	65.2	0.60	0.30
Body oblique length	74.5	74.0	0.64	0.46
Chest circumference	85.5	87.0	0.60	0.04
Cannon circumference	11.8	11.7	0.08	0.26

DMI = dry matter intake; ADG = average daily gain; SEM = standard error of the mean.



**Fig. 1.** Effect of sodium butyrate on the development of rumen epithelium. A: Rumen cranial abdomen sac epithelium of the SB group. B: Rumen cranial abdomen sac epithelium of the Ctrl group. C: The single rumen epithelium papillae of the SB group. D: The single rumen epithelium papillae of the Ctrl group. E: The rumen papillae length between SB and Ctrl. F: The rumen papillae width between SB and Ctrl. G: The rumen papillae surface area between SB and Ctrl. The scale bar of C and D is 1 mm. \*P < 0.05.

**Table 3**

Effect of dietary sodium butyrate supplementation on the rumen microbial fermentation of preweaning bull calves.

Item	Treatment		SEM	P-value
	Control (Ctrl)	Sodium butyrate (SB)		
pH	6.59	6.65	0.123	0.80
NH <sub>3</sub> -N, mmol/L	12.34	11.20	0.578	0.39
Total SCFA, mmol/L	57.88	64.94	6.626	0.62
Acetate, mmol/L	36.68	38.69	3.424	0.78
Propionate, mmol/L	13.10	16.24	1.958	0.45
Butyrate, mmol/L	4.21	12.22	1.429	<0.01
Acetate to propionate ratio	2.82	2.59	0.115	0.33

**Table 4**

Effect of dietary sodium butyrate supplementation on the gastrointestinal development of preweaning bull calves.

Item	Treatment		SEM	P-value
	Control (Ctrl)	Sodium butyrate (SB)		
<b>Forestomach</b>				
Rumen volume, L	6.89	8.40	0.609	0.26
Rumen-reticulum weight, kg	1.04	1.39	0.088	0.04
Omasum weight, kg	0.24	0.30	0.021	0.23
Abomasum weight, kg	0.34	0.40	0.016	0.07
<b>Total intestine</b>				
Weight, kg	4.91	5.57	0.211	0.12
Length, m	26.97	31.50	1.268	<0.01
<b>Duodenum</b>				
Weight, kg	0.42	0.47	0.102	0.10
Length, m	0.67	0.67	0.032	0.99
<b>Jejunum</b>				
Weight, kg	1.65	2.07	0.087	0.01
Length, m	21.26	24.94	0.770	0.01
<b>Ileum</b>				
Weight, kg	0.24	0.27	0.015	0.38
Length, m	1.31	1.69	0.114	0.09
<b>Cecum</b>				
Weight, kg	0.12	0.13	0.006	0.49
Length, m	0.34	0.35	0.018	0.65
<b>Colon</b>				
Weight, kg	0.36	0.40	0.022	0.28
Length, m	2.73	3.11	0.115	0.10
<b>Rectum</b>				
Weight, kg	0.43	0.53	0.046	0.31
Length, m	0.66	0.72	0.021	0.16

### 3.4. Transcriptomic analysis of rumen and jejunum epithelium

A total of 23 samples, including 12 jejunum (5 in Ctrl and 7 in SB) and 11 rumen samples (6 in Ctrl and 5 in SB), had a successful RNA extraction and sequencing and produced 756.24 M clean reads. The average output of each sample was 32.88 M. A total of 23,657 genes were detected.

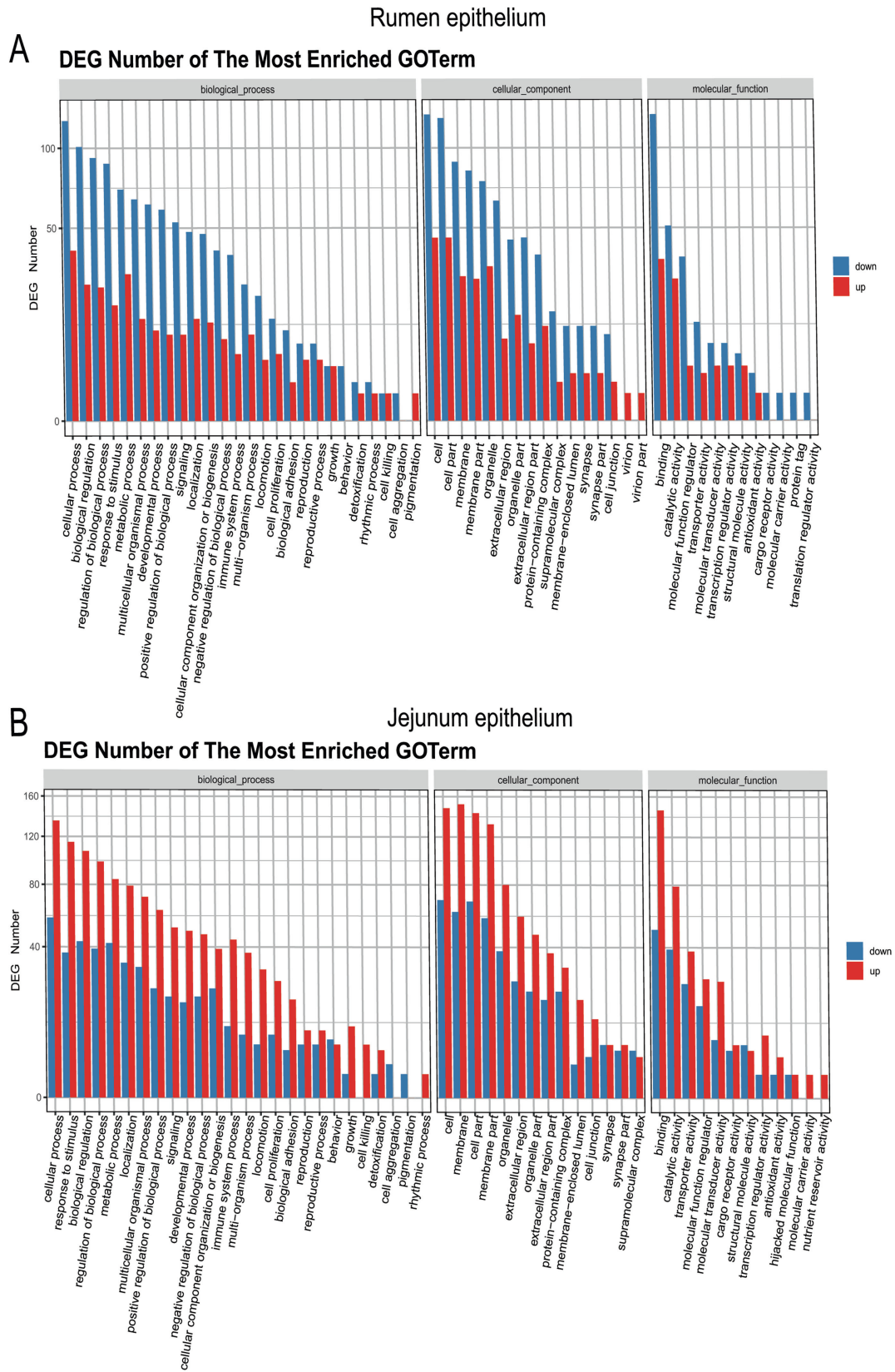
In the SB group, 139 up-regulated genes and 317 down-regulated genes were identified in rumen epithelium ( $P < 0.05$ ; Fig. 2A), and 401 up-regulated genes and 186 down-regulated genes were identified in jejunum epithelium ( $P < 0.05$ ; Fig. 2B). The KEGG enrichment analysis showed that the SB supplementation suppressed the NF-kappa B signaling pathway (DEG: *PPKCB*, *CXCL8*, *CCL19*, *CXCL12*), interleukin-17 signaling pathway (DEG: *IL17A*, *IL17B*, *CXCL2*, *CXCL10*, *CXCL5*, *CCL20*, *MMP9*), chemokine signaling pathway (DEG: *CCL20*, *CXCL2*, *CXCL12*, *CXCL5*, *CXCL10*, *CCL2*, *CCL4*, *CCL8*), and cytokine–cytokine receptor interaction (DEG: *IL17A*, *IL17B*, *CCL19*, *CCL4*, *CCL8*, *CXCL1*, *CXCL5*, *CXCL10*, *CXCL12*) (Fig. 3A, Fig. 4A) in the rumen epithelium. In the jejunum epithelium, the SB supplementation activated arachidonic acid metabolism (DEG: *CYP4A11*, *PLA2G2F*, *AKR1C3*, *ALOX12E*), synthesis and degradation of ketone bodies (DEG: *HMGCS2*, *BDH1*, *LOC100295719*), the immune network for immunoglobulin A (IgA) production (DEG: *BOLA-DQB*, *CD28*, *LOC100295645*) and nitrogen

metabolism (DEG: *CA1*, *CA2*, *CA3*, *CA12*) (Figs. 3B and 4B), and also influenced the PPAR signaling pathway (DEG: *FABP4*, *FABP6*, *CYP4A11*, *PCK1*, *FABP1*, *APOA1*, *CD36*), fat digestion and absorption (DEG: *PLA2G2F*, *APOA1*, *APOA4*, *APOB*, *CD36*, *FABP1*), the IL-17 signaling pathway (DEG: *FOSL1*, *CXCL10*, *MMP13*), and cytokine–cytokine receptor interaction (DEG: *CXCL9*, *CXCL10*, *CXCL11*, *CXCL13*, *IL34*, *IL36A*). The change of genes in the transcriptomic analysis was in accordance with the results of qPCR (Fig. 4C and D).

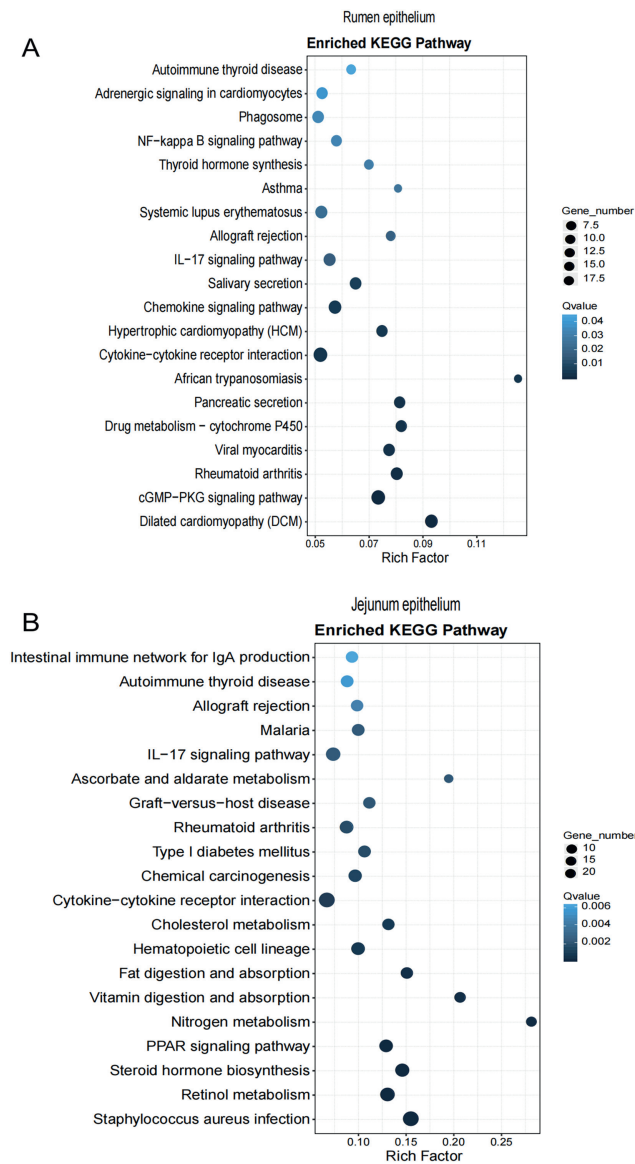
### 3.5. Rumen microbial diversity and function

A total of 10 rumen digesta samples produced 441.89 Gbp raw bases, 428.72 Gbp clean bases, and 3.26 Mbp contigs. The average length and N50 of contigs were 1,769 bp and 4,776 bp, respectively. The nonredundant gene set contained 3.13 Mbp genes, and the mapped ratio of reads in each sample was more than 70%. The number of nonredundant genes annotated by GO, KEGG, eggNOG, NR and Swiss-Prot databases was 1.58 Mbp (50.5%), 1.76 Mbp (56.2%), 2.45 Mbp (78.3%), 2.65 Mbp (84.9%), and 1.53 Mbp (48.9%), respectively.

In both Ctrl and SB groups, the top 4 dominant genera were *Prevotella*, *Ruminococcus*, unclassified\_order\_Bacteroidales, unclassified\_family\_Prevotellaceae (Fig. S1). Butyrate supplementation had



**Fig. 2.** Effect of sodium butyrate on GO pathway of rumen epithelium and jejunum. A: Number of differentially expressed genes (up and down) and Gene Ontology term enrichment in rumen samples. B: Number of differentially expressed genes (up and down) and Gene Ontology term enrichment in jejunum samples. The red bar represents up-regulated genes. The blue bar represents down-regulated genes. DEG = differentially expressed genes.



**Fig. 3.** The effect of sodium butyrate on KEGG pathways of rumen epithelium and jejunum. A: The enriched KEGG pathways in rumen samples. B: The enriched KEGG pathways in jejunum samples. The size of the circle represents the number of genes. The larger circle means more genes. The color of the circle represents the significance of the pathway enrichment. The darker color means stronger significance.

no significant influence on Shannon diversity ( $P > 0.05$ ) (Fig. S2) but a clear separation of microbial composition could be observed between the Ctrl and SB groups in principal coordinate analysis ( $P < 0.05$ ; Fig. 5A), showing that SB influenced microbial community structure. Butyrate greatly improved the relative abundance of *Clostridium citroniae*, *Bacillus subtilis*, *Cordyceps confragosa*, *Eubacterium limosum*, and *Pelotomaculum thermopropionicum* ( $P < 0.05$ ; Fig. 5B). As for the functional genes, most of the differentially expressed genes ( $P < 0.05$ ) were mainly enriched to the pathways related to carbohydrate metabolism, such as fructose and mannose metabolism, glycolysis and pyruvate metabolism, and galactose metabolism (Fig. 6). Further analysis of the differences in carbohydrate-active enzymes between the Ctrl and SB groups found that the top 30 enriched carbohydrate-active enzymes mainly belonged to glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), and carbohydrate-binding modules

(CBM), and the number of GH, GT, PL, and CBM in the SB group were also much more than those in the Ctrl group ( $P < 0.05$ ; Fig. 7).

## 4. Discussion

### 4.1. Growth and gastrointestinal tract development

In most studies reported in the literature, SB was supplemented to starter feeds containing less than 20% NDF (Górka et al., 2011a, 2011b; Nazari et al., 2012). In this study, we supplemented SB to a starter feed with about 30% NDF and found a promoting effect on growth and GIT development. When NDF is higher in a starter ration, less butyrate is released by ruminal and gut bacteria. As a consequence, more SB is needed in the diet to meet the butyrate requirements of calves.

In this study, SB supplementation in the starter notably increased DMI, ADG, body weight, and carcass weight. These results are consistent with the study by Ślusarczyk et al. (2010) who supplemented SB at 0.3%, 1%, and 3% DM to the calf diet and found an increased starter intake and ADG at 0.3% and 1% levels during postweaning. The improved ADG, body weight, and carcass weight may be partially explained by the increased DMI.

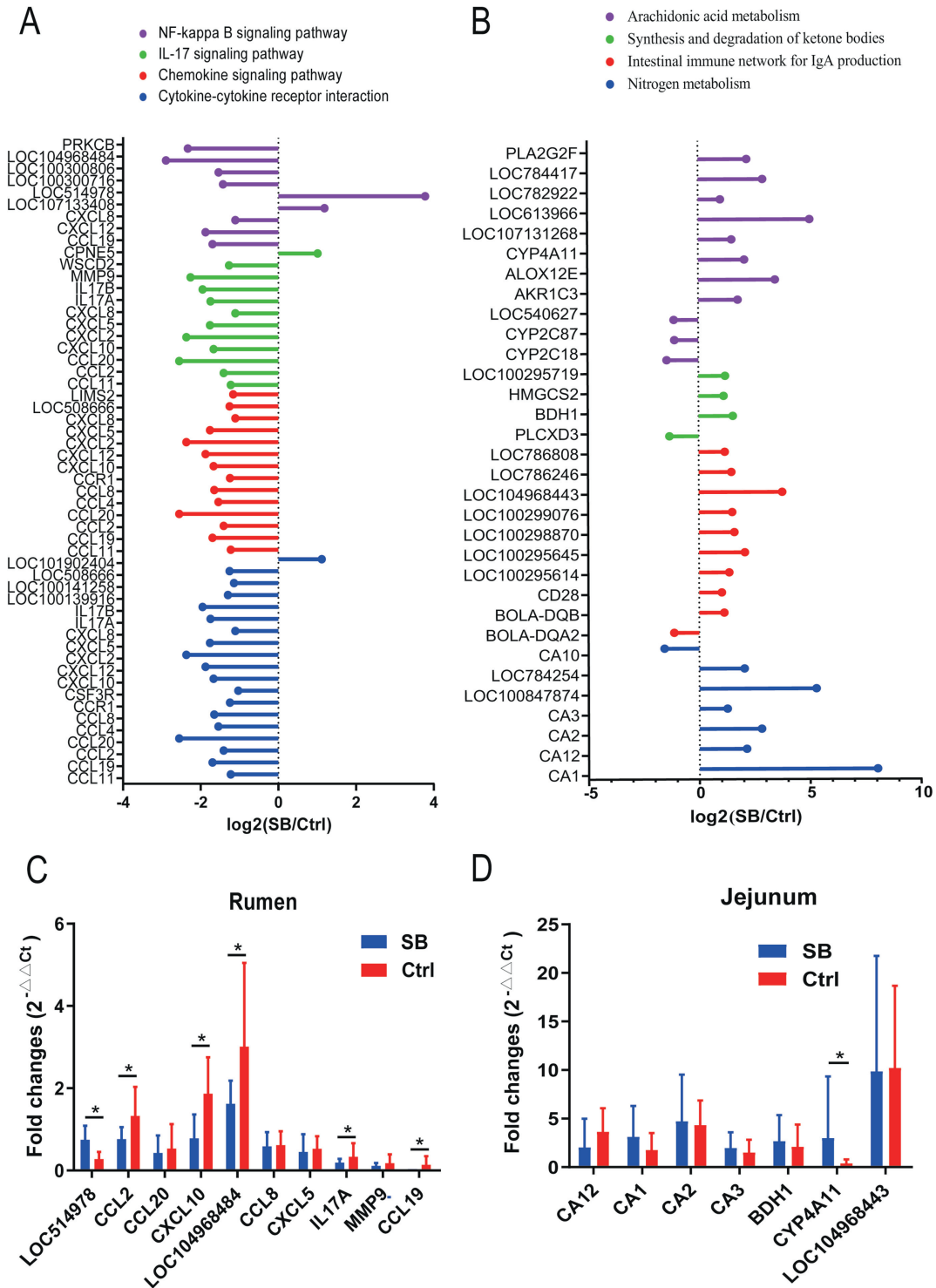
Butyrate supplied in feed promoted rumen epithelium development, maintained the health of the GIT, and improved the growth of calves in the pre-ruminant stage (Górka et al., 2011b; Niwinska et al., 2017). Butyrate reaches the rumen first and is largely absorbed through the rumen epithelium with little passing out of the omasum; therefore, butyrate supplementation has a weaker effect on the distal intestine compared to the proximal intestine (Meale et al., 2017). Our results showed that the weights of the rumen-reticulum, abomasum, and jejunum and the length of rumen papillae were significantly increased, but the weights of the ileum, colon, and cecum were not influenced, which is consistent with the results of other studies with the dietary NDF content of 40% (Górka et al., 2018a, 2018b). It is well-known that the GIT plays an important role in nutrient digestion and absorption, therefore the improved development of the rumen and jejunum potentially leads to an improved rate of weight gain in calves. Our data on ADG of calves also support this hypothesis.

However, some studies showed either no or negative effects of SB supplementation on the growth of calves. Wanat et al. (2015) found that the supplementation of SB at 0.3%, 0.6%, and 0.9% of the diet had a negative effect on feed intake and calf growth performance. Burakowska et al. (2021a, 2021b) indicated that supplementation of SB had little effect on the growth of calves and a negative effect on rumen development but in their studies, microencapsulated SB was used. The method of delivery (Górka et al., 2011a, 2014), nutrient composition of basal diets (Górka et al., 2011b), or supplemental dose (Liu et al., 2021) all influenced the effects of SB supplementation. These factors might be reasons for the discrepancy between this study and some of the previous studies.

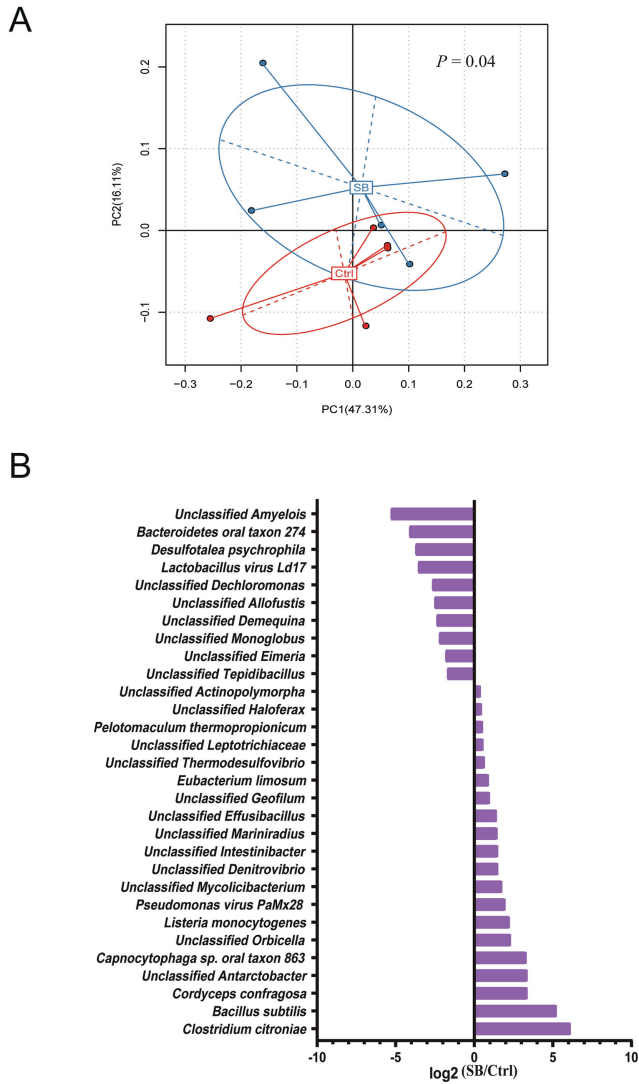
As for rumen fermentation, butyrate concentration was significantly increased, which was consistent with the result of Liu et al. (2021). The increase in butyrate may be related to the rapid dissociation of butyrate from sodium butyrate in the starter in the rumen.

### 4.2. Gene expression of gastrointestinal tract epithelium

Calves undergo 3 stages of physiological changes in their digestive system after birth, including extrauterine life, the pre-ruminant stage and weaning. At birth, the calf's GIT is not developed well and cannot digest solid feed (Baldwin et al., 2004). The development of the GIT is important for calf health and growth



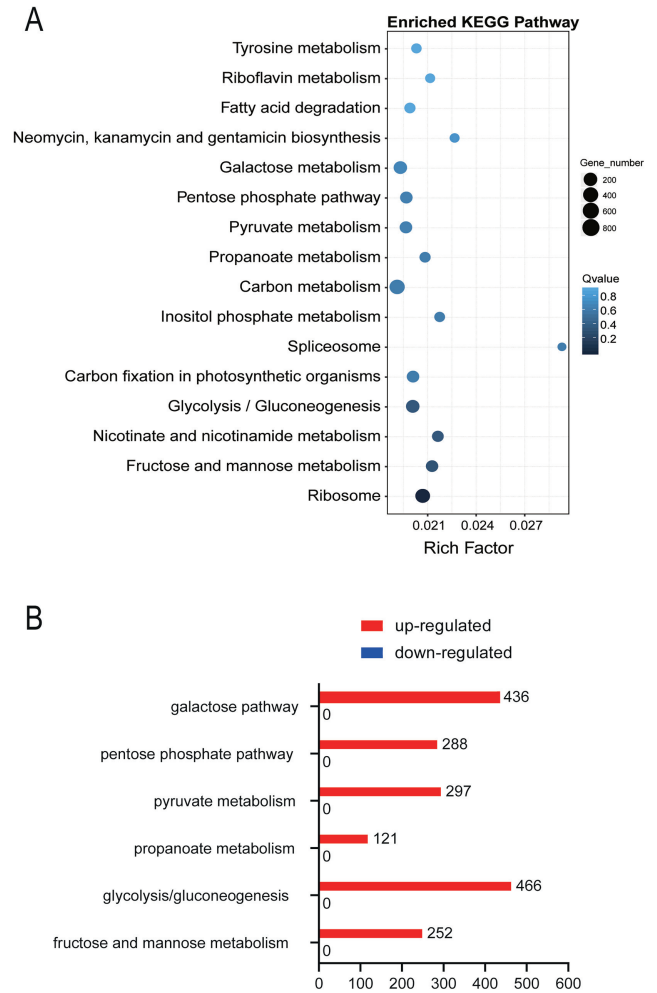
**Fig. 4.** The verification of differentially expressed genes of enrichment pathways in the rumen and jejunum. A: The differentially expressed genes of 4 enrichment pathways related to immunology and inflammation in rumen samples. The purple represents the NF-Kappa B signaling pathway. The green represents the IL-17 signaling pathway. The red represents the chemokine signaling pathway. The blue represents the cytokine–cytokine signaling pathway. B: The differentially expressed genes of 4 enrichment pathways related to immunology and energy metabolism in jejunum samples. The purple represents the fat digestion and absorption signaling pathway. The green represents the synthesis and degradation of ketone bodies signaling pathway. The red represents the intestinal immune network for IgA production signaling pathway. The blue represents the nitrogen metabolism signaling pathway. C: The qPCR results of differentially expressed genes in the rumen. D: The qPCR results of differentially expressed genes in the jejunum. The x axis shows the fold change of genes (SB vs Ctrl). The y axis shows the significant genes of the top 4 enrichment pathways (A and B). \* $P < 0.05$ .



**Fig. 5.** The effect of sodium butyrate (SB) on the composition of the rumen microbiome. A: Principal coordinate analysis of rumen bacterial samples on species level. B: The top 30 different species between SB and Ctrl groups effected by sodium butyrate.

(Meale et al., 2017), and have a long-term effect on their subsequent growth, development, and production performance (Meale et al., 2017).

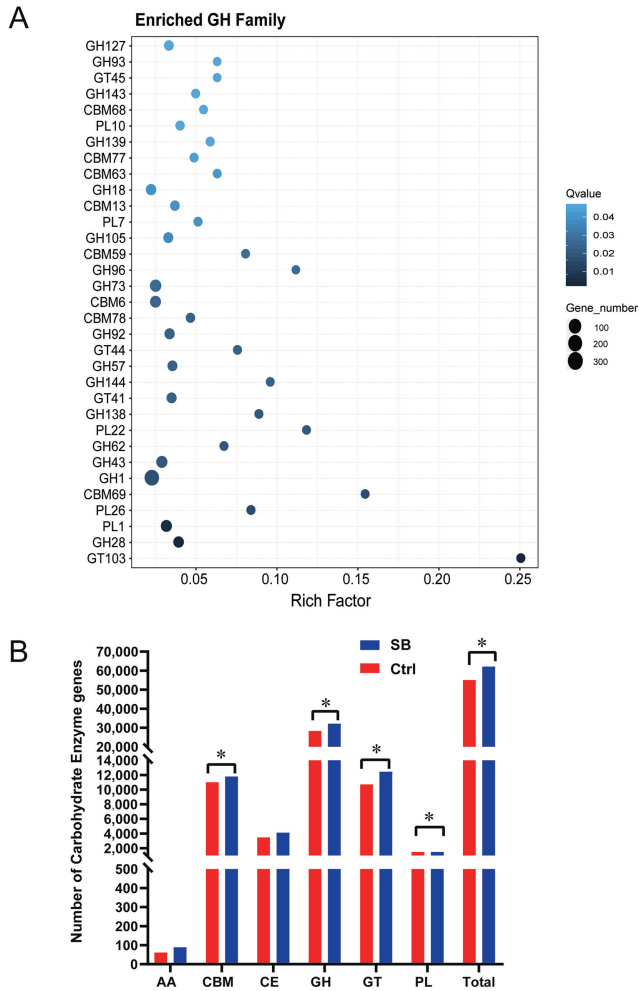
The gene transcription and expression of epithelium cells are crucial for the development of the GIT. In the present study, the RNAseq was used to identify differentially expressed genes and affected pathways. Only the epithelium of the rumen and jejunum were analysed because other segments of the gut were not found to have significant changes in length when SB was supplemented. The results showed that the signaling pathways related to inflammation, such as the NF- $\kappa$ B signaling pathway, IL-17 signaling pathway, chemokine signaling pathway, and cytokine–cytokine signaling pathway were down-regulated, indicating that the calves were in better health. IL-17 is a pro-inflammatory factor mainly produced by T helper cell 17 (Th17) which is also named CD4<sup>+</sup> (Amatya et al., 2017). It activates the NF- $\kappa$ B signaling pathway, induces the production of chemokines C-X-C motif chemokine 1 (CXCL1), CXCL5, promotes the secretion of pro-inflammatory factor IL-6, and finally causes the development of chronic inflammation or tumors (Zhao et al., 2020). Oral administration of butyrate inhibited the NF- $\kappa$ B signaling pathway to relieve symptoms of colitis in mice (Lee et al.,



**Fig. 6.** The effect of sodium butyrate on the KEGG metabolic pathways of the rumen microbiome. A: The top 20 enriched KEGG pathways of the rumen microbiome. B: The number of changed genes in pathways related to carbohydrate metabolism.

2017), and also reduced the differentiation and function of Th17 to relieve the colitis by inhibiting the secretion of IL-17 (Ploger et al., 2012). NF- $\kappa$ B is a central inflammatory mediator, and it increases the production of IL-6, CXCL1, CXCL10 and matrix metalloproteinases (MMP) to induce inflammation (Liu et al., 2017). The addition of SB can restrain the IL-17 signaling pathway, NF- $\kappa$ B signaling pathway, chemokine signaling pathway, and chemokine–chemokine signaling pathway by inhibiting the expression of *IL-17A*, *IL-17B*, *CCL2*, *CCL20*, *CCL19*, *CCL11*, *CXCL2*, *CXCL5*, *CXCL8*, *MMP9*, which could inhibit inflammation and promote the healthy development of the GIT.

Meanwhile, we found that the intestinal immune network for the IgA production signaling pathway was up-regulated when induced by SB in the rumen epithelium, which showed that calves had stronger immunity. Under the normal physiological conditions, IgA is the main immunoglobulin subtype in the intestinal mucosa and neutralizes bacterial toxins on the surface of the epithelium (Tezuka and Ohteki, 2019). In mice, butyrate promoted the production of non-dependent T cell IgA to maintain intestinal immune homeostasis (Isobe et al., 2020). Furthermore, the arachidonic acid (ARA) metabolism signaling pathway was also regulated by SB. The metabolism of ARA was related to inflammation, and there were 3 main metabolic pathways including cyclooxygenase, lipoxygenase (LOX), and cytochrome P450 (CYP450) (Wang et al., 2021). Butyrate



**Fig. 7.** The effect of sodium butyrate (SB) on the number of rumen microbial carbohydrate-active enzymes. A: The enriched carbohydrate-active enzymes of the rumen microbiome. B: The number of differential carbohydrate-active enzymes in different modules. AA = auxiliary activities; CBM = carbohydrate-binding lyases; CE = carbohydrate esterases; GH = glycoside hydrolases; GT = glycosyl transferases; PL = polysaccharide lyases. \* $P < 0.05$ .

mainly regulated CYP metabolism and cyclooxygenase metabolism by up-regulating *CYP4A11*, *PLA2G2F*, *AKR1C3*, *ALOX12E* and down-regulating *CYP2C18* and *CYP2C87* to regulate the ARA metabolism signaling pathway. Arachidonic acid derivatives were involved in many pathologies, but it should be noted that the harmful effects of ARA were mostly shown in non-physiological conditions, and ARA seemed to be contained in infant formula and may be beneficial to the growth and health of infants (Calder, 2007). In addition, the optimal fecal score percentage of calves was also significantly improved. Fecal score cannot provide definitive answers to nutritional questions but is a consequence of metabolic end products. It is the simplest way to evaluate GIT health.

The synthesis of ketone bodies (acetoacetate, 3-hydroxybutyrate, acetone) was promoted by up-regulating the gene abundance of hydroxy-methyl-glutaryl-CoA synthase, hydroxy-methyl-glutaryl-CoA lyase and 3-hydroxybutyrate dehydrogenase. Ketone bodies are an energy source in the rumen epithelium, especially when glucose is limited (Akram, 2013; Laffel, 1999). Supplemented butyrate also regulated lipid metabolism by up-regulating *PLA2G2F*, *FABP4*, *FABP6* genes and down-regulating *FABP1*, *APOA1*, *APOA4* and *APOB* genes. The fatty acid binding protein (FABP) family participates in the binding and transportation of

fatty acids, CD36, and FABP constitute the perception machinery of the fatty acid system (Straub, 2020). *FABP4* was found to be up-regulated during lactation (Bionaz and Looor, 2008) and was also related to fat deposition in Australian cattle *longissimus* (Barendse et al., 2009). *FABP1* and *FABP6* were up-regulated to promote fatty acid uptake and intracellular transport after parturition (Ghaffari et al., 2021). In addition, apolipoproteins (including apoA and apoB) participated in lipid transportation during lipid metabolism.

### 4.3. Ruminal microbial community structure and function

The rumen is the first organ of the GIT that SB flowed into, and it also had the highest SB content. The inhabited microbial community in the rumen had cross-interaction with butyrate. In this study, the metagenomic sequencing technology was adopted to investigate the influence of SB on the rumen microbiome. The top 4 dominant genera in the rumen were *Prevotella*, *Ruminococcus*, unclassified Bacteroidales and unclassified Prevotellaceae, which was similar to the previous studies (Darwin et al., 2017; Li et al., 2012) indicating the reliability of metagenomic sequencing. Among these genera, *Prevotella* and unclassified Prevotellaceae are highly efficient in hemicellulose degradation, and *Ruminococcus* is highly efficient in cellulose degradation (Koike and Kobayashi, 2009; Mizrahi et al., 2021). Species belonging to *Bacteroidales* harbor extensive saccharolytic ability (Salysers et al., 1977).

In this study, the approach of shotgun instead of 16S rRNA gene sequencing was adopted to obtain bacterial profile at the species level. The results showed the dietary supplementation of SB altered microbial composition patterns and increased the relative abundance of *C. citroniae*, *B. subtilis*, *C. confragosa*, *E. limosum*, and *P. thermopropionicum*. *C. citroniae* can produce beta-glucosidase, xylanase, and urease and thus can utilize cellulose, hemicellulose, and urea (Nie et al., 2020). *B. subtilis* is a common probiotic that has been shown to promote the development of rumen papillae, increased the rate of rumen development of calves during both weaning and post-weaning periods (Sun et al., 2011), and enhanced the immunity by inducing the secretion of IgG (Sun et al., 2010). *E. limosum* is a type of acetogen that takes advantage of hydrogen, carbon monoxide, and carbon dioxide to form acetate and butyrate (Jeong et al., 2015). *P. thermopropionicum* is a typical syntrophic bacterium that converts propionate, butyrate, lactate, and ethanol produced by other bacteria to acetate, carbon dioxide, hydrogen, and formate which are in turn substrates for methanogens (Kato et al., 2009). The increased relative abundance of these species with SB supplementation indicated that the carbohydrate or SCFA metabolism pathways of the ruminal microbial community may be activated.

To further explore microbial community functions, we analyzed the relative abundance of functional genes. The results showed that the microbial genes related to carbohydrate metabolism were greatly influenced by SB. The relevant pathways, including fructose and mannose metabolism, glycolysis and pyruvate metabolism, and galactose metabolism, were up-regulated, and the numbers of genes in GH1, GH28, PL1, and GH43 were greatly enriched. In the rumen, carbohydrates are mostly converted into SCFA by microbiota and used for energy by ruminants (Chai et al., 2021). Enzymes from GH1 are capable of degrading cellulose, those from GH28 and PL1 are responsible for pectin degradation, and those from GH43 have the ability to degrade arabinoxylan (Flint et al., 2012). It was indicated that butyrate strengthened the ruminal microbial fermentation of lignocellulose and pectin in calves and the metabolites, e.g. SCFA, serve as the primary source of energy or nutrients for the GIT or host development (Darwin et al., 2017). In the rumen, the microbial metabolites or nutrients also had a relationship with epithelial physiological function. The development of

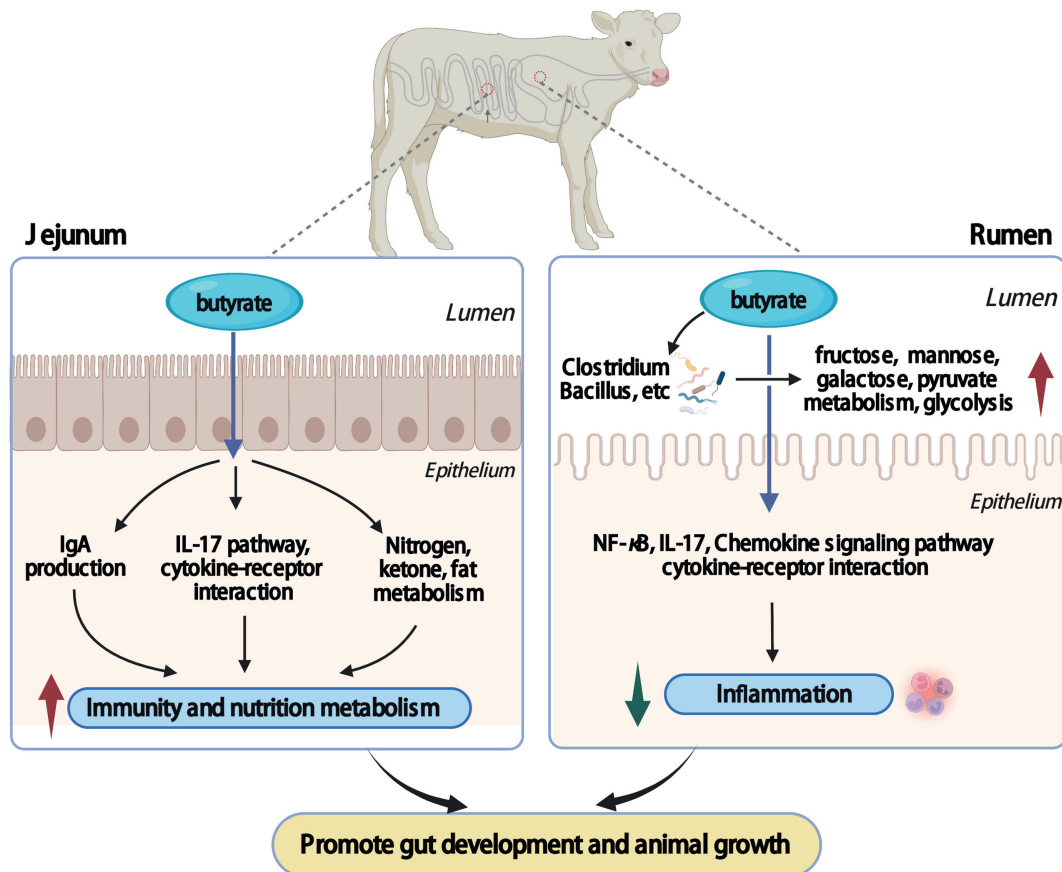


Fig. 8. The overview diagram of effect of butyrate on the rumen, rumen microbiome and jejunum.

epithelial inflammation is closely related to nutrient metabolism. The production of inflammatory factors consumes energy, and some cytokines regulate both inflammation and nutrient metabolism (Roche, 2021). The inhibition of inflammation not only reduces energy expenditure but also increases feed intake (Wang and Ye, 2015). The interaction between inflammation and nutrient metabolism can be regulated by nutrient composition in the diet (Roche, 2021). For instance, Kundi et al. (2021) found that a diet rich in fiber regulates metabolism to relieve inflammation. The microbial metabolites induced by SB also have the potential to influence the function of the epithelium, but this new hypothesis needs to be verified in future studies.

## 5. Conclusion

Supplementation of SB in the starter diet of pre-weaned dairy bull calves improved the growth and GIT development. SB down-regulated the IL-17 and NF-κB signaling pathways, inhibited the secretion of chemokines and cytokines, and reduced the inflammation of the rumen and jejunum epithelium. SB also influenced ruminal microbial community and activated nutrients such as carbohydrate metabolism pathways. Overall, butyrate exhibited promising effects on growth and GIT development by inhibiting inflammation, promoting immunity, and activating ruminal microbial metabolism (Fig. 8).

## Author contributions

**Shengguo Zhao:** Conceptualization, Methodology, Data curation, Writing-Reviewing and Editing. **Huiyue Zhong:** Investigation,

Writing-Original Draft, Visualization. **Wenjing Yu:** Investigation. **Xuezhao Sun:** Writing-Review and Editing. **Min Wang:** Conceptualization, Methodology, Writing-Reviewing and Editing. **Bo Lin:** Conceptualization, Methodology. **Nan Zheng:** Conceptualization, Methodology. **Jiaqi Wang:** Conceptualization, Methodology.

## Availability of data and material

The raw data of metagenomic sequencing was submitted to China National GeneBank DataBase (CNP0003181).

## 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.

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## Appendix supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2023.04.004>.

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