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Original article

Supplementation of tributyrin improves the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets

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SUMMARY

Background & aims: Intrauterine growth restriction (IUGR) neonates suffer from growth restriction. Tributyrin (TB), a pro-drug of butyrate, can facilitate the growth of animals. This study was to investigate the effects of TB supplementation on the growth of IUGR neonatal piglets.

Methods: Sixteen IUGR and 8 NBW (normal body weight) neonatal piglets were chosen, weaned at 7th day and fed basic milk diets (NBW and IUGR group) or the basic diets supplemented with 0.1% tributyrin (IT group, IUGR piglets fed with tributyrin) until day 21 (n = 8). The body weights of the piglets on days 0, 7, 10, 14, 17, and 20 were measured. The digestive enzyme activity, intestinal morphology, immunoglobulin levels and gene expression of IgG, FcRn and GPR41 in the small intestines were analyzed.

Results: The body weights of the piglets in the IUGR and IT group were similar, and both were lower than the NBW group on days 10 and 14. However, after day 17, the IT group exhibited improved ($P < 0.05$) body weights compared to that of the IUGR group. The piglets were sacrificed on day 21. Compared with the NBW piglets, IUGR impaired the development of immune organs and small intestines, impaired the intestinal villus morphology, decreased ($P < 0.05$) most of the tested intestinal digestive enzyme activities, decreased ($P < 0.05$) the ileal sIgA and IgG levels, and down-regulated ($P < 0.05$) the intestinal IgG and GPR41 expression. Piglets in the IT group exhibited a better-developed ($P < 0.05$) spleen and small intestines, improved intestinal villus morphology, increased ($P < 0.05$) intestinal villus surface areas, enhanced ($P < 0.05$) digestive enzyme activities, and up-regulated ($P < 0.05$) expression of IgG and GPR41 mRNA compared to those of the IUGR group.

Conclusions: TB supplementation improves the growth and the intestinal digestive and barrier functions in IUGR piglets during the suckling period.

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1. Introduction

Intrauterine growth restriction (IUGR) is a serious problem in human infants and animals. Pigs are multifetal animals and exhibit serious IUGR occurrence [1]. IUGR significantly affects the neonatal survival, postnatal growth, immunity, and life-long health of the animals [2]. Although better management techniques and mammalian nutrient regulation have substantially helped improve complications, IUGR continues to be a significant problem because the knowledge of the effect of nutrition on the mechanisms modulating neonatal infants' growth is still deficient [3]. IUGR can significantly affect the development and barrier functions of the intestine in neonates and may cause long-term growth impairment [4]. Studies on the effects of new nutrients on IUGR may be helpful in solving the complications that are associated with IUGR.

Tributyrin (TB), a triglyceride with 3 butyrate glyceryl-tributyrates, can act as a butyrate pro-drug [5]. Butyrate, a kind of

Abbreviations: NBW, normal body weight; IUGR, intrauterine growth restriction; TB, tributyrin; IT, IUGR piglets that were fed TB-supplemented milk diets; SCFA, short-chain fatty acid; SI, small intestine; MLN, mesenteric lymph nodes; sIgA, secretory Immunoglobulin A; IgG, Immunoglobulin G; FcRn, Fc receptor; GPR41, G-protein-coupled receptor 41 (also called free fatty acid receptor 3 or FFAR3).

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short-chain fatty acid (SCFA), can protect the intestine by inhibiting the growth of bacteria, increasing mucosal cell proliferation, and improving intestinal cell development and colonic defense work [6]. Butyrate can also has an effect on the immunity and has anti-inflammatory effects [7]. In addition, pre-weaning supplementation with sodium butyrate is more efficient in stimulating feed intake and body growth compared with post-weaning supplementation [8]. A study on weaned piglets showed that the supplementation of sodium butyrate only improved the beginning phase of the piglets' adaption to the solid diets [9]. Another study of weaned piglets also showed that sodium butyrate addition to the diets does not increase the body growth but may modulate their immunity [7]. The use of TB can reduce the drawbacks of butyrate; TB is better orally tolerated than the butyrate and has been accepted as a food additive [10,11]. The role of TB for suckling piglets is not clear at present, and the effects of TB on IUGR for both humans and livestock have not been studied. The aim of the study was to explore the effects of suckling period TB addition on the body growth and the intestinal digestive and barrier functions of IUGR neonatal piglets.

2. Materials and methods

2.1. Piglets and diets

The experiment was conducted on a trial pig farm that was owned by the Anyou Company (Xuancheng, Anhui Province, China). Neonatal Duroc × (Landrace × Yorkshire) piglets were chosen from eight sows. The sows were all with the birth order of the 3rd or 4th and were fed the same gestating diet, which met the National Research Council (NRC, 2012) nutrient requirements [12]. In each litter, two IUGR and one normal-body-weight piglets were obtained. Piglets with birth body weights of 0.95 kg (SD 0.08) and 1.56 kg (SD 0.07) were defined as IUGR and NBW piglets, respectively [13]. The NBW piglets were allocated to the NBW (normal birth weight) group, and the two IUGR piglets from one litter were randomly assigned to the IUGR and IT (IUGR with TB supplementation) groups (n = 8/group, 4 males and 4 females in each group). The piglets in the NBW and IUGR group were fed basic milk diets, while the piglets in the IT group were fed basic milk diets supplemented with 0.1% tributyrin from 7 to 21 days after birth (the composition of the diets and the nutrient content are shown in Table 1). Tributyrin (casein-coated; the content of TB was 50%) was supplied for free by the Xin' ao Company (Xia Men, China). Six piglets (n = 6/group, 3 males and 3 females) in each group were selected randomly and sacrificed for further study.

All procedures of the animal experiment were accepted by the Nanjing Agricultural University's Institutional Animal Care and Use Committee, People's Republic of China. The piglets were weighed on days 0, 7, 11, 14, 17, and 21. The piglets were fed a warm milk substitute (milk bubbled with warm water, 100 g of milk in 600 ml of water) ad libitum every 2–3 h (calculated according to the total body weight of the piglets in each treatment, about 75 ml per kg of body weight) approximately 9–10 times every day. We measured the milk that was supplied and the milk that remained in each group with a plastic volumetric cylinder and calculated the absolute intake (intake = supplied-remaining) at each feeding time from days 11–20. We further converted this volume to the weight of the milk substitute taken by the formula: milk density = weight/volume. We calculated the ADG (average body weight gain of a day), ADFI (average feed intake of a day), and the gain-to-feed ratio from days 11–20; however, we obtained the data of the means of each group but no SEM (standard error mean). Because we intended to imitate the natural feeding conditions for the piglets, we fed them approximately 10 times each day for 14 days. To save time and to imitate the natural feeding conditions more effectively, we did not use bottle feeding, which requires that each piglet be fed individually. Instead, we taught the piglets how to drink the warm milk from plates and fed all of the piglets at the same time. The disadvantage of this was that we could only obtain the average feed intake in one group but not the feed intake for each piglet.

Table 1
Composition and nutrient content of the diets (DM basis).

Items	Control	TB[a]
Ingredients (g/kg)		
Whey protein concentrate (34% CP)	300	300
Milk fat powder (11% CP)	350	350
Whole milk powder	200	200
α-Casein	50	50
Glucose	60	60
Pre-mixed mixture[b]	40	40
TB	0	1
Nutrient content[c]		
CP (%)	23.31	23.28
Digestible energy (MJ/kg)	14.88	14.88
Lysine (%)	1.51	1.51
Methionine (%)	0.43	0.43
Threonine (%)	0.89	0.89
Tryptophan (%)	0.25	0.25
Ca (%)	0.85	0.85
Total P (%)	0.70	0.71

CP, crude protein; TB, tributyrin; Ca calcium; P phosphorus.
[a] In the TB diets, 0.1% TB was added to the control diet. Tributyrin (casein coated; net content of TB is 50%) was supplied for free by the Xin' ao Company in Xia Men. The two diets were isonitrogenous and isoenergetic.
[b] The main contents of the pre-mixed mixture (per kg of the pre-mixed mixture): Cu (as $CuSO_4 \cdot 5H_2O$) 600 mg; Fe ($FeSO_4 \cdot 7H_2O$) 8400 mg; Mn ($MnSO_4 \cdot H_2O$) 315 mg; Se (as Na_2SeO_3) 17 mg; Zn ($ZnSO_4 \cdot 7H_2O$) 12,500 mg; Vitamin A 55,000 IU; Vitamin D 5500 IU; Vitamin E 400 IU; Vitamin K 12.5 mg; Biotin 2 mg; Choline 15 mg; Folacin 7.5 mg; Riboflavin 100 mg; Thiamin 317.5 mg; Vitamin B_6 175 mg; Vitamin B_{12} 500 mg.
[c] The nutrient contents of CP, Ca, and P were the examined values, the contents of other nutrient were calculated according to the contents of the nutrient of the ingredients referring to NRC (2012) [15].

The number of piglets who had diarrhea was recorded during the experimental period (from days 11–20), and the diarrhea rates were calculated using the following formula: diarrhea rate = the sum of diarrhea piglets on each day during the experiment ÷ (the number of the experimental piglets × the number of experimental days) × 100%. The piglets in the three groups (NBW, IUGR and IT group) were raised in three separate little plastic houses (1.5 m × 0.7 m × 0.7 m; environmentally controlled; the ambient temperature is 33 °C) and could have water freely. The piglets that were selected for slaughter were stunned by electric shock and killed by jugular bloodletting at the end of the experimental period.

2.2. Sample collections

The 18 piglets (from the NBW, IUGR, and IT groups; 6 piglets per group) were weighed prior to euthanization. The SI was taken out from the abdominal cavity immediately after the animal died and was divided into the duodenum (about first 10-cm segment after the stomach), the jejunum (about half of the small intestine below the duodenum), and the ileum (the left part of the small intestine). The weight and length of the emptied SI with its mesenteric attachments removed were measured. Two samples of about 1 cm in length from each section were collected. One sample was fixed in 4% buffered formaldehyde; the other sample was fixed in 2.5% buffered glutaraldehyde. The contents from the three segments of the SI were collected and stored in a −20 °C freezer for digestive enzyme activity testing. The mucosa scrapings of the small

intestines were also collected and saved in the environment of liquid N_2 for the gene expression analysis and were stored in a −20 °C freezer for the double-antibody enzyme-linked immunosorbent assay (ELISA) analysis of the immunoglobulins. The thymus, spleen, MLNs (mesentery-dissected mesenteric lymph nodes along the entire SI), and small intestines were collected and weighed; the ratio of the organ weights to the body weights and the relative length of each segment of the SI were also calculated.

2.3. Histological study

Small sections of the SI that were fixed in 2.5% buffered glutaraldehyde were used for scanning electron microscopy studies [14]. These sections were cleaned with phosphate-buffered saline and subsequently fixed in 1% osmium tetroxide (2 h,at the temperature of 4 °C). The samples were then dehydrated with an growing gradient of acetone for 2 h at a room temperature (approximately 18 °C), and the critical-point of the tissues were then dried in a 3-methylbutyl (isoamyl) acetate. The dried samples were lastly coated with gold–palladium and a digital scanning electron microscope (S-3000N, Hitachi High-Technologies Corp., Japan) was used to observe these samples.

The SI samples fixed in 4% buffered formaldehyde were dried up using a graded series of xylene and ethanol. After that, they were embedded in paraffin for further study. The samples (5 microns in size) were then deparaffinized using xylene and rehydrated with graded dilutions of ethanol. The slides were stained with hematoxylin and eosin (HE). Five to ten slides for each tissue (the middle site of the three segments of the small intestine) were prepared, and the images were acquired using an optical binocular microscope (Olympus BX5; Olympus) that was coupled with a digital camera (Nikon H5500L). The morphological measurements of the villus length (v), crypt depth, villus width (w), muscle thickness, and mucosa thickness were calculated using the Image-Pro Plus (IPP) software, and the villus area (S) was calculated using the following formula: $S = \pi \times \frac{w}{2}\sqrt{\left(\frac{w}{2}\right)^2 + v^2}$.

2.4. Digestive enzyme activity analysis

The frozen contents of the three segments of the small intestine were homogenized with a hand-held homogenizer (PRO 200; PRO SCIENTIFIC) in 1 ml of cold PBS (pH = 7.4, 0.01 M). The homogenate was centrifuged (500 × g, (10 min at 4 °C), and the supernatants were collected. The activity levels of the digestive enzymes lactase (Lactase Activity Testing Kit, No: A082-1), sucrase (Sucrase Activity Testing Kit, No: A082-2), maltase (Maltase Activity Testing Kit, No: A082-3), lipase (Lipase Activity Testing Kit, No: A054), and trypsin (Trypsin Activity Testing Kit, No: A080-2) were determined according to the manufacturer's instructions of Nanjing JianCheng Bioengineering Institute (Nanjing, Jiangsu, China). The samples were assayed in triplicate. The amount of protein in the intestinal contents was measured using the bicinchoninic acid (BCA) protein assay of Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). The results were obtained by using a microplate reader and were displayed as U per milligram of protein (for lactase, sucrase, maltase, and trypsin) or U per gram of protein (for lipase). U is the international unit of the enzyme, which is the amount of the enzyme that is required to convert 1 μmol of substrate in 1 min.

2.5. Immunoglobulin level analysis by ELISA

The frozen intestinal mucosa from the jejunum and ileum (200 mg) was homogenized with a hand-held homogenizer (PRO 200; PRO SCIENTIFIC) in 1 ml of cold PBS (pH = 7.4, 0.01 M). The homogenate was centrifuged (1200 × g, 15 min at 4 °C), and the supernatants were collected. The levels of immunoglobulin (IgG and Secretory Immunoglobulin A) in the duodenum, jejunum, and ileum were determined using ELISA (pig-specific, No: H106 & No: H108) according to the manufacturer's (Nanjing JianCheng Bioengineering Institute, Jiangsu, China) instructions. The samples were assayed in triplicate. The protein contents of the mucosal tissues were measured using the BCA (Beyotime Institute of Biotechnology, Jiangsu, China) protein assay. The ELISA results were obtained by a microplate reader at the light length of 450 nm and were displayed as milligrams per gram of protein.

2.6. mRNA expression analysis

The total RNA was got from the mucosa of the duodenum, jejunum, and ileum by using TRIzol (Invitrogen, Shanghai, China). The RNA was quantified spectrophotometrically based on the light absorption at 260 nm (A260) or 280 nm. The RNA content in each sample was estimated with the formula: RNA content = A260 × 40 (μg/ml) × dilution factor. The quality of the RNA was evaluated with agarose gel electrophoresis. 1 μg RNA of each sample was used to synthesize corresponding cDNA in a 20-μl reaction system according to the instructions of the Primer-Script™ Reagent Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China).

The primers of gene-specific that were used are listed in Table 2 and were synthesized using Invitrogen Biotech Co. Ltd. (Shanghai, China). β-Actin and GAPDH were chosen as housekeeping genes (similar results were obtained and only showed the results using β-Actin as a normalizer). The RT-PCR (real-time polymerase chain reaction) assays were conducted using the ABI Step-One-Plus RT-PCR system (Applied Biosystems, Foster City, CA) and the SYBR Premix Ex Taq™ Kit (TaKaRa Biotechnology Co. Ltd, Dalian, China). The following thermocycle was used for the RT-PCR: 95 °C (30 s), 95 °C (40 cycles, 5 s for each cycle) and 60 °C (31 s). We also performed a melting curve cycle analysis and compared the amplification efficiency of the target genes with that of the housekeeping genes. The melt curves of each gene had one single and one overlapping peak, and the amplification efficiency for each target gene and housekeeping gene were similar and all within the span of 0.9–1.1 (shown in Table 2). The relative gene expression levels were determined using Step-One software (Applied Bio-systems) and were calculated with the $2^{-\Delta\Delta CT}$ method relative to the items in the duodenum of the NBW group [15].

2.7. Statistical analysis

For the body weight data the modeling of repeated records (8 repeats) was conducted using a mixed model with day (D), group (G), and D × G as fixed effects and piglets as random effects. As the NBW group, IUGR group, and IUGR + TB group were paired within litters, the difference between any two groups were analysized by paired *t* test. The data of the intestinal morphology, digestive enzyme activity, immunoglobulin levels, and gene expression levels were also analyzed as a mixed model with group (G), intestine (I), and G × I as fixed effects and the piglets as random effects by the MIXED procedure. If the *P* value of the interaction G × I was less than 0.05, One-Way ANOVA was conducted among the different groups in each segment of the intestines. All data were analysized by using SPSS 17.0. The statistical difference was significant while the *P* value was no more than 0.05. Datas are expressed as the means with their mean standard errors. The mean values in a row had different superscripts a or b are of significant difference ($P < 0.05$).

Table 2
Primer sequences used in quantitative real time-PCR assays.[a]

Gene	Accession no.	Forward primer 5′-3′ (F) Reverse primer 5′-3′ (R)		Amplicon size, bp	Amplification efficiency
FcRn	NM_214197.2	F	CCTTGGATCTCTTTTCATGGGG	199	1.00
		R	CAGAGGCTCAGAAATACCAGC		
IgG	NM_213828.1	F	GTCAGGGCTCTACTCCCTCA	123	1.03
		R	GTTCCAACACGCTTGTCCAC		
GPR41 (FFAR3)	JX566878.1	F	CTCATCACCAGCTACTGCCG	208	1.01
		R	AATTCAGGGTGCTGAGGAGC		
β-actin	XM 003357928	F	TGCGGGACATCAAGGAGAAG	216	1.00
		R	AGTTGAAGGTGGTCTCGTGG		
GAPDH	NM001206359.1	F	CGTCCCTGAGACACGATGGT	194	1.00
		R	GCCTTGACTGTGCCGTGGAAT		

[a] IgG, Immunoglobulin G; FcRn, Fc receptor; GPR41, G-protein-coupled receptor 41 (also called free fatty acid receptor 3 or FFAR3).

3. Results

3.1. Growth performance and development of immune organs and SI

The interaction effect (group × day) was not significant ($P > 0.05$) (**data not shown**). The body weights of the piglets in the three groups were highest in the NBW group, lower in the IT group, and lowest in the IUGR group ($P < 0.001$) (**data not shown**). The body weights of all of the piglets increased as the piglets grew ($P < 0.001$) (**data not shown**). The body weights of the piglets in the IUGR and IT groups were lower ($P < 0.05$) than those in the NBW group on days 0, 7, 11, and 14. On days 17 and 20, the IT group exhibited improved body weight compared with that of the IUGR group ($P < 0.05$), and the body weights of the piglets in the IT group were closer to those of the NBW group ($P > 0.05$) (Fig. 1a). We also calculated the ADG, ADFI, and the gain-to-feed ratio from days 11–20. The ADG (g/d) of the NBW, IUGR, and IT groups was 174, 58, and 196, respectively. The ADFI (g/d) of the three groups was 321, 100, and 238, respectively. The gain-to-feed ratio was 0.54, 0.58, and 0.82, respectively. The diarrhea rate of the NBW, IUGR, and IT groups was 0.89%, 2.68%, and 0.89%, respectively.

Table 3 indicates that the relative weight of the spleen in the IT group was higher than that of the IUGR group ($P < 0.05$). The jejunum was thinner in the IUGR group compared with that of the NBW group ($P < 0.05$); however, the relative lengths of the duodenum in the IUGR group were longer ($P < 0.05$) compared with those of the NBW group, while the IT group had a similar ($P > 0.05$) relative length of the duodenum and the jejunum compared with the NBW group and had a lower ($P < 0.05$) relative length of the jejunum than that of the IUGR group.

3.2. Histological observation of the small intestine

Figure 2 shows that the villi of the SI (middle site of the whole small intestine) in the IUGR group were jagged and shorter compared with those of the NBW and IT groups. The villi in the SI of the IUGR group tended to be compressed and easily impaired, while the villi in the SIs of the IT and NBW groups were integrated and well developed.

There were no interaction effects between the groups and the intestines for the villus length, villus width, muscle thickness, or mucosa thickness. The villus length of the SI was highest in the NBW group, lower in the IT group, and lowest in the IUGR group ($P < 0.05$); the muscular thickness and the mucosa thickness were highest in the IT group, lower in the NBW group, and lowest in the IUGR group ($P < 0.01$). In addition, the duodenum and jejunum were the key sites of villus morphological differences. In the duodenum, a shorter villus length ($P < 0.05$), a higher crypt depth ($P < 0.05$), a lower VCR ($P < 0.05$), a thinner mucosal layer ($P < 0.05$), and a smaller ($P < 0.05$) villus surface area were observed in the IUGR group compared with those of the NBW group, while the IT group had an increased ($P < 0.05$) villus length, villus width, VCR, muscle thickness, mucosa thickness, and villus surface area and a decreased ($P < 0.05$) crypt depth compared with those of the IUGR group. In the jejunum, the IT group also had an increased ($P < 0.05$) villus length, VCR, mucosa thickness, and villus surface area compared with that of the IUGR group. Compared with the NBW group, the IT group had a higher ($P < 0.05$) VCR in the jejunum and a similar ($P > 0.05$) villus surface area in both the duodenum and the jejunum (Table 4).

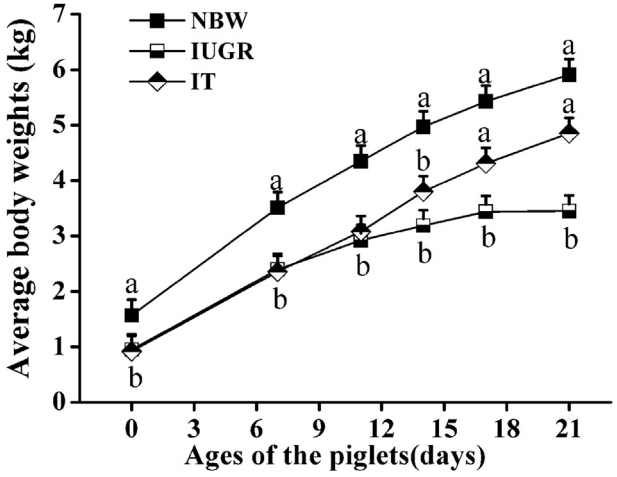


Fig. 1. Body weights of the IUGR piglets that were supplemented with TB during the suckling period. Eight litters of newborn piglets were selected. Two IUGR piglets and one normal body weightpiglets were chose from each litter. The piglets in the NBW and IUGR group were fed milk basic diets, while the piglets in the IT group (IUGR piglets fed with tributyrin) fed with the basic milk diets supplemented with 0.1% tributyrin from 7 to 21 days of age. The data are expressed as the mean ± SEM, n = 8. The data of the body weights of the piglets were collected using a mixed model with day (D), group (G) and D × G as fixed effects and piglets as a random effect. The difference between any two groups was analysized by the paired *t* test. The interaction between the treatment and day was not significant ($P > 0.05$). The effect of the group was significant ($P < 0.001$); the body weights of the piglets in the three groups were highest in the NBW group, lower in the IT group and lowest in the IUGR group ($P < 0.01$) (data not shown). The effect of the day was significant ($P < 0.001$); the body weights of all of the piglets increased with age (data not shown). The average body weights of the piglets in the IUGR and IT groups were lower ($P < 0.05$) than those of the NBW group on days 0, 7, 11, and 14. However, on days 17 and 20, the IT group exhibited improved body weight compared to that of the IUGR group ($P < 0.05$), and the body weights of the piglets in the IT group were closer to those of the NBW group ($P > 0.05$) (Fig. 1).

Table 3
The effects of TB supplementation during the suckling period on the development of the SI and immune organs in IUGR piglets.

MLN	Group			SEM
	NBW[a]	IUGR[a]	IT[a]	
Body weight (g)	5.74a	3.89b	5.23a	0.28
Spleen weight (g)	10.64a	5.15b	11.44a	1.12
MLN[b] weight (g)	11.24	7.82	10.91	1.13
Length of duodenum (cm)	27.14	27.28	29.28	2.05
Weight of duodenum (g)	7.43	5.71	8.33	0.72
Length of jejunum (cm)	376.80	319.40	338.64	24.98
Weight of jejunum (g)	123.28	83.89	106.96	11.32
Weight of ileum (g)	113.08	81.61	115.95	11.29
Relative weight of spleen (g/kg)	1.83ab	1.34b	2.17a	0.18
Relative weight of MLN[b] (g/kg)	1.94	2.00	2.09	0.20
Relative[c] length of duodenum (cm/kg)	4.74b	7.09a	5.64ab	0.43
Duodenum weight/duodenum length (g/cm)	0.29	0.21	0.29	0.03
Relative length of jejunum (cm/kg)	65.88ab	81.95a	64.81b	3.49
Jejunum weight/jejunum length (g/cm)	0.33a	0.26b	0.31ab	0.10
Ileum weight/ileum length (g/cm)	0.30	0.26	0.35	0.04

All values are means ± SEM, n = 6; SEM, standard error mean.
a,b: Mean values within a row with different online a or b are with significantly different ($P < 0.05$).
[a] NBW, normal piglets that were fed control milk diets; IUGR, IUGR piglets that were fed control milk diets; IT, IUGR piglets that were fed tributyrin-supplemented diets.
[b] MLN, mesenteric lymph nodes.
[c] Relative means of the ratio of the item versus the body weight.

3.3. Digestive enzyme activity levels in the SI

There was an interaction effect between the intestines and groups ($P < 0.01$) for all of the tested enzyme activities. In the duodenum, the IUGR group had lower ($P < 0.05$) levels of lactase, sucrase, and lipase but a higher ($P < 0.05$) level of maltase than the NBW group, while the IT group had increased ($P < 0.05$) amounts of lactase compared with those of the IUGR group. In the jejunum, the activity of trypsin was lower ($P < 0.05$), but the activity of sucrase was higher ($P < 0.05$) in the IUGR group than that in the NBW group, whereas the IT group had an increased ($P < 0.05$) level of trypsin and a decreased ($P < 0.05$) level of sucrase compared with those in the IUGR group. In the ileum, the IUGR group had lower ($P < 0.05$) levels of five tested digestive enzymes compared with those of the NBW group, whereas the IT group had higher ($P < 0.05$) amounts of the five tested digestive enzymes compared with those of the IUGR group and even higher ($P < 0.05$) lactase, maltase, lipase, and trypsin levels than those of the NBW group (Table 5).

3.4. Immunoglobulin levels

Table 6 shows that there were no interaction effects between the groups and the intestines on the sIgA and IgG levels. The sIgA and IgG levels in the ileal mucosa of the IUGR piglets were lower ($P < 0.05$) than that of the NBW group, while the IT group had higher ($P < 0.05$) levels of both immunoglobulins compared with the IUGR group and a lower ($P < 0.05$) level of IgG in the ileum compared with the NBW group.

3.5. Relative gene expression of FcRn, IgG, and GPR41

The relative gene expression of FcRn was site dependent ($P < 0.05$) and highest in the duodenum. The expression of IgG in the three segments of the small intestine in the IUGR group was lower ($P < 0.05$) than that in the NBW group, but the IT group had an increased ($P < 0.05$) IgG expression in the ileum. The GPR41 expression in the duodenum and ileum was down-regulated ($P < 0.05$) in the IUGR group compared with the NBW group, whereas the GPR41 expression in the IT group was up-regulated ($P < 0.05$) in the duodenum and ileum compared with the IUGR group (Table 7).

4. Discussion

IUGR neonates suffer from the mal-development of the intestines, especially during the early post-natal period. Tributyrin is a well-tolerated oral pro-drug of butyrate that can facilitate the growth performance of animals [16]. We hypothesized that the administration of TB during the suckling period may improve the growth performance of IUGR piglets by enhancing the digestive and barrier functions of the small intestine. As far as we know, this is the first research to explore the effects of TB supplementation to the diets on the body growth, SI development, and digestive and barrier functions of the SI using IUGR suckling piglets.

Tributyrin (TB) is a butyrate pro-drug [5]. Butyrate is a kind of short chain fatty acid which is predominantly created by bacterias in the intestine through the anaerobic fermentation of dietary fibers in vivo [17]. A previous study on neonatal piglets demonstrated that the supplementation of sodium butyrate increased daily body weight gain of the piglets compared with that of the control group [18], but a study on weaned piglets indicated that the addition of sodium butyrate improved only the beginning phase of the weanling piglets' adaption to the solid diet [9]. Another study on weaned piglets also showed that dietary sodium butyrate supplementation does not facilitate the growth performance [7]; however, Lu et al. (2008) found that sodium butyrate is useful in improving the body weight gain of weanling pigs [19]. In addition, pre-weaning supplementation with sodium butyrate is more efficient in stimulating body growth and feed intake compared to post-weaning supplementation [8]. These differing results may have occurred because of different amounts of added butyrate and different lasting times of the experiments. After the supplementation of TB for IUGR suckling piglets for only 10 days, their body weights became significantly heavier than those of the IUGR piglets that were fed without TB, and their body weights even became close to those of the NBW piglets. This result may be due to an increased ADFI, an increased gain-to-feed ratio, and a lowered diarrhea rate in the IT group compared with the IUGR group. These results indicate that TB was quite helpful in facilitating the growth of IUGR neonatal piglets.

TB supplementation also improved the development of the spleen in IUGR piglets. Previous studies have also reported that sodium butyrate can enhance the weight of the spleen in broilers

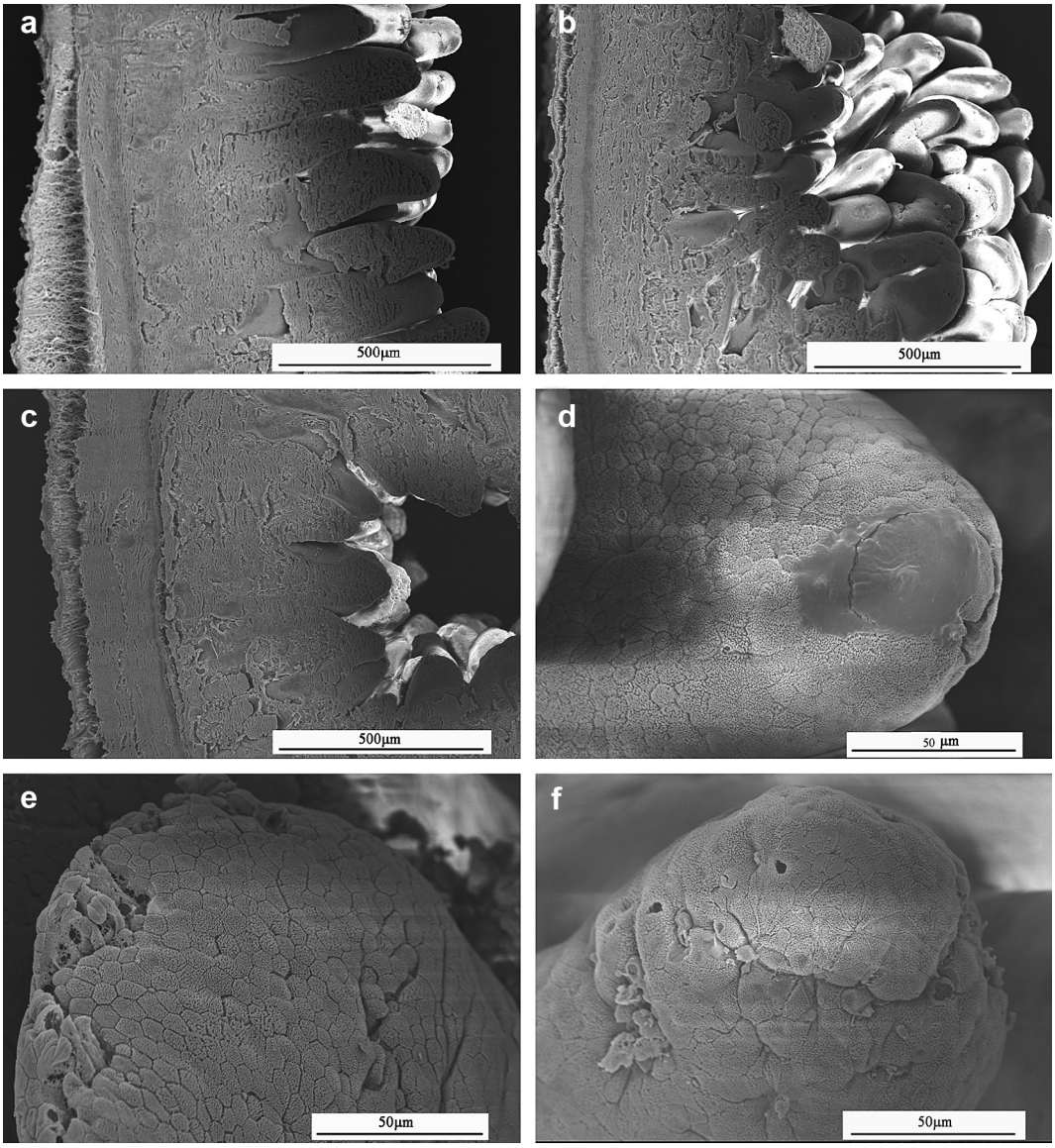


Fig. 2. Intestinal villus morphology of the piglets in the NBW, IUGR and IT groups. The villus morphology in the small intestine (middle site of the small intestine) of piglets (NBW: a and d; IUGR: b and e; IT: c and f) was observed by scanning electron microscopy (S-3000N; Hitachi High-Technologies Corp; Japan). The villi of the SI (middle site of the whole small intestine) in the IUGR group (Fig. 2**b**) were jagged and shorter compared to those the NBW (Fig. 2**a**) and IT (Fig. 2**c**) groups. The villi in the SI of the IUGR (Fig. 2**e**) group tended to be compressed and impaired, while the villi in the SI of the IT (Fig. 2**f**) and NBW (Fig. 2**d**) groups were integrated and well developed. Scale bars: 500 μm in a, b and c; 50 μm in d, e and f. NBW, normal body weight; IUGR, intrauterine growth restriction; IT, IUGR piglets fed TB-supplemented milk diets.

[4]. Although the effects of TB supplementation on the function of the spleen in IUGR piglets are not clear at present (ongoing research), the improved development of the spleen in the IT groups indicates an improved immunity for IUGR piglets. Studies of neonatal piglets have suggested that IUGR is associated with a poorly developed SI that is shorter, thinner, and relatively longer than that in NBW piglets [13,20,21]. In this study, the IUGR piglets still had a relatively longer duodenum and jejunum and a thinner jejunum at 21 days than did the NBW piglets. The TB supplementation modified the mal-development of the SI in the piglets of the IUGR group. These results suggest that feeding TB to IUGR piglets during the suckling period stimulates the development of both their immune organs and small intestines.

Intestinal morphology is an important factor reflecting intestinal health and function in pigs [20]. The jagged and metamorphotic villi and the reduced villus heights that were observed in the IUGR piglets indicate a decreased ability to absorb nutrients as well as a vulnerable mechanical barrier in the SIs of IUGR piglets. Supplementation with TB facilitated the recovery of the mechanical barrier and absorptive functions of the SI; a healthier intestinal villus and a larger villus surface area were also observed. A previous study suggested that butyrate may be trophic to the SI in developing infants, in infants who have short bowel syndrome, and in infants who are recovering from malnutrition [21]. One study suggested that sodium butyrate supplementation may improve the jejunal and ileal mucosa development in formula-fed piglets [18]; however, in this study, the effects of TB on the morphology of the SI villi were mainly on the duodenum and jejunum. Tributyrin is a triglyceride with a 3 butyrate glyceryl-tributyrate; thus, the tributyrin is highly digested by the intestinal lipase in the upper part of the small intestine. Therefore, a rapid absorption of butyric acid by the duodenum and jejunum mucosa is expected; however, further

Table 4
The effects of TB supplementation during the suckling period on villus morphology of the SI in IUGR piglets.

Intestine (I)	Group (G)[a]			Mean	SEM	*P* Value		
	NBW	IUGR	IT			G	I	G × I
Villus length (μm)								
Duodenum	567a	401b	512ab	493	10	0.019	<0.001	0.075
Jejunum	407	390	451	416				
Ileum	341	328	337	335				
Mean	439	373	433	415				
Villus width (μm)								
Duodenum	115	103	119	112	1.7	0.143	<0.001	0.216
Jejunum	92	89	103	95				
Ileum	93	94	89	92				
Mean	100	95	103	100				
Crypt depth (μm)								
Duodenum	85b	103a	70c	86	0.98	<0.001	<0.001	<0.001
Jejunum	51	51	52	51				
Ileum	46	51	50	49				
Mean	61	68	57	62				
Villus length/crypt depth								
Duodenum	6.7a	3.9b	7.4a	6.0	0.12	<0.001	<0.001	<0.001
Jejunum	8.0ab	7.6b	8.8a	8.1				
Ileum	7.4	6.4	6.7	6.9				
Mean	7.3	6.0	7.6	7.0				
Muscle thickness (μm)								
Duodenum	125b	102b	175a	134	3.6	0.001	<0.001	0.106
Jejunum	88	87	106	93				
Ileum	109	104	120	111				
Mean	107	97	133	113				
Mucosa thickness (μm)								
Duodenum	825a	667b	803a	765	13	0.027	<0.001	0.356
Jejunum	629	609	679	639				
Ileum	542	505	538	529				
Mean	665	594	673	644				
Villus surface area (mm^2)								
Duodenum	0.104a	0.065b	0.097a	0.089	0.002	0.012	<0.001	0.041
Jejunum	0.060	0.055	0.074	0.063				
Ileum	0.050	0.049	0.047	0.049				
Mean	0.072	0.056	0.073	0.067				

a,b: Mean values within a row with different online a or b are with significantly different ($P < 0.05$).
[a] NBW, normal piglets fed with control milk diets; IUGR, IUGR piglets fed with control milk diets; IT, IUGR piglets fed with tributyrin supplemented diets; I, intestine; SEM, standard error mean.

studies are needed to identify where the oral-fed TB begins to be digested and absorbed. TB also showed trophic effects on the smooth muscles in the SI, suggesting an enhanced small intestinal motility that could thereby change the conditions of feed passage and absorption. The changes of the morphology and muscle thickness in the SI of the IUGR piglets that were supplemented with TB led to an improved ability to absorb nutrients and enhanced the mechanical barrier function of the SI, which facilitated the growth of the IUGR piglets.

IUGR inhibited the activity of the five tested digestive enzymes (lactase, sucrase, maltase, lipase, and trypsin) in the ileum of the IUGR piglets. TB supplementation facilitated the activation of these enzymes, and the lactase, maltase, lipase, and trypsin activity levels were even greater in the IT piglets compared to those in the NBW piglets. IUGR also inhibited the levels of lactase, sucrase, and lipase in the duodenum and the level of trypsin in the jejunum, while TB supplementation improved the activities of these enzymes. Some studies have reported that fat coating may target butyrate in the small intestine, improving the digestive and absorptive capacities in pigs [22]. The activity of the digestive enzymes also helps maintain the intestinal barrier function during bacterial invasion [20]. The increased digestive enzyme activity levels complemented the enzymes that were lacking in the IUGR piglets and thus improved the nutritive value of their feedstuffs; however, IUGR increased the level of maltase in the duodenum and increased the level of sucrase in the jejunum; the IT group had similar amounts of these two enzymes compared with those of the NBW group. This result is beyond our hypothesis and is difficult to explain at present; however, the average level of sucrase in the three sites of the small intestine was still lowest in the IUGR group, higher in the NBW group, and highest in the IT group. The improved growth of the IUGR piglets that were supplemented with TB could also be due to the increased digestive enzyme activity levels, which demonstrated increased digestive ability and improved chemical barrier functions in the SI.

Butyrate has a beneficial effect on the immunity of both humans and rodents and exerts an anti-inflammatory effect both in vivo and in vitro [23]. Our previous studies have demonstrated that IUGR impairs SI immunity in neonatal piglets, and the ileum is the key site of impairment [24]. In this study, the levels of sIgA and IgG in the ileum were also reduced in the IUGR piglets, and supplementation with TB enhanced the ileal secretion of both immunoglobulins. In this study, IUGR also depressed the relative IgG expression level in the jejunum and ileum. The ability of piglets to acquire IgG by transcytosis ceases by 24–36 h after birth; thus, the IgG levels that were measured in this study were produced by the piglets. Our results suggest an enhanced ability to secrete IgG in the ileum after TB supplementation in the IUGR piglets. FcRn plays an important role in neonates by transferring IgG from the mother or from the colostrum to the newborn. The relative expression level of FcRn was

Table 5
The effects of TB supplementation during the suckling period on digestive enzyme activity levels in the SI of IUGR piglets.

Items	Group (G)			SEM	*P* Value		
	NBW[a]	IUGR[a]	IT[a]		G	I[a]	G × I
Lactase (U[a]/mg protein)							
Duodenum	71b	30c	110a	6.5	<0.001	<0.001	<0.001
Jejunum	151b	157ab	213a				
Ileum	197b	176b	463a				
Sucrase (U/mg protein)							
Duodenum	90a	63b	60b	7.6	0.010	<0.001	<0.001
Jejunum	217b	340a	236b				
Ileum	442a	169b	389a				
Maltase (U/mg protein)							
Duodenum	135b	202a	224ab	13	<0.001	<0.001	<0.001
Jejunum	427	410	541				
Ileum	710b	460c	1116a				
Lipase (U/g protein)							
Duodenum	438a	152b	184b	36	<0.001	<0.001	<0.001
Jejunum	879	723	803				
Ileum	836b	425c	1763a				
Trypsin (U/mg protein)							
Duodenum	68	99	79	54	<0.001	<0.001	<0.001
Jejunum	2048a	489b	1381a				
Ileum	2684b	393c	4136a				

a,b,c: Mean values within a row with different online a, b or c are with significantly different ($P < 0.05$).

[a] NBW, normal piglets fed with control milk diets; IUGR, IUGR piglets fed with control milk diets; IT, IUGR piglets fed with tributyrin supplemented diets; I, intestine; SEM, standard error mean; U, international unit of the enzyme; the amount of the enzyme needed to make the conversion of 1 micromles substrate within 1 min.

not significantly affected by IUGR or TB supplementation. This result is in contrast to our hypothesis, and further research is required in this area.

GPR41, an orphan G protein-coupled receptor, is an active receptor of butyrate [25,26]. Previous studies have demonstrated that SCFAs activate intestinal GPR41 and thus mediate tissue inflammation and protective immunity in mice [27]. In this study, IUGR depressed the expression of GPR41; after TB addition, the level of GPR41 expression was up-regulated in the ileum. The activation of GPR41 can be a compensatory mechanism in order to counter the up-regulated histone H3 acetylation levels that caused by butyrate and may induce the proliferation of cells [28]. Our results suggest that TB supplementation might enhance the intestinal development and immunity of IUGR piglets by activating the expression of GPR41.

Table 6
The effects of TB supplementation during the suckling period on immunoglobulin levels in the mucosa of the SI in IUGR piglets.

Intestine(I)	Group (G)[a]			Mean	SEM	*P* Value		
	NBW	IUGR	IT			G	I	G × I
sIgA[b] (mg/g protein)								
Jejunum	7.8	5.1	8.1	7.0	0.52	0.115	0.012	0.832
Ileum	5.2a	2.9b	4.4ab	4.2				
Mean	6.5	4.0	6.3	5.6				
IgG[b] (mg/g protein)								
Jejunum	322	325	385	344	22	0.244	0.061	0.199
Ileum	351a	174b	253ab	259				
Mean	336	250	319	302				

a,b: Mean values within a row with different online a or b are with significantly different ($P < 0.05$).

[a] NBW, normal piglets fed with control milk diets; IUGR, IUGR piglets fed with control milk diets; IT, IUGR piglets fed with tributyrin supplemented diets; I, intestine; SEM, standard error mean.

[b] IgA, stimulatory Immunoglobulin A; IgG, Immunoglobulin G.

Table 7
The effects of TB supplementation during the suckling period on the relative gene expression of IgG and its receptor GPR41 in the small intestine of IUGR piglets.

MLN	Group			SEM
	NBW[a]	IUGR[a]	IT[a]	
FcRn				
Duodenum	1.012	0.802	0.843	0.080
Jejunum	0.529	0.582	0.451	0.060
Ileum	0.626	0.467	0.475	0.060
IgG[a]				
Duodenum	1.019a	0.535b	0.417b	0.050
Jejunum	0.132a	0.023b	0.039b	0.005
Ileum	0.281a	0.029c	0.114b	0.010
GPR41[b]				
Duodenum	1.020a	0.068b	0.191b	0.044
Jejunum	0.071a	0.040b	0.050b	0.006
Ileum	0.106a	0.050b	0.129a	0.008

All values are means ± SEM, n = 6; SEM, standard error mean.

a,b,c: Mean values within a row with different online a, b or c are with significantly different ($P < 0.05$).

[a] NBW, normal piglets fed with control milk diets; IUGR, IUGR piglets fed with control milk diets; IT, IUGR piglets fed with tributyrin supplemented diets; I, intestine.

[b] FcRn, Fc receptor; IgG, immunoglobulin G; GPR41, G-protein-coupled receptor 41 (also called free fatty acid receptor 3 or FFAR3).

In conclusion, TB supplementation during the suckling period facilitated body weight gain, improved the development of immune organs and small intestines, improved intestinal villus morphology, increased the intestinal villus surface area and the intestinal digestive enzymes activity, increased the mucosal immunoglobulin levels, and up-regulated the gene expression of IgG and GPR41 in the ileum of IUGR piglets. As the structure and physiology of the intestines are very similar between human and pig, IUGR neonatal piglets can also be a very good model for the human research in the intestinal structure and function [29]. Our study indicated a new method to treat IUGR shortly after birth through dietary supplementation with tributyrin during the suckling period. However, more researches are needed if we need to adapt this to human clinical nutrition.

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Conflict of interest

No conflict of interest exits in the submission of this manuscript.

Authors contribution

Li Dong made the planning, execution, data analysis and paper writing. Xiang Zhong and Tian Wang helps the planning, analysis and revision. The other co-authors (Jintian He, Lili Zhang, Kaiwen Bai and Wen Xu) have all helped in the experimental conduction and manuscript revision. Tian Wang had primary responsibility for the final content. All authors have read and approved the final manuscript.

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