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Original Research Article

Benefits of tributyrin on growth performance, gastrointestinal tract development, ruminal bacteria and volatile fatty acid formation of weaned Small-Tailed Han lambs

Zhiwei Li [a, 1], Xueer Wang [b, 1], Wei Wang [a], Ran An [a], Yaxin Wang [a], Qingchang Ren [a, c, *], Jingjing Xuan [d, *]

[a] College of Animal Science, Anhui Science and Technology University, Fengyang, 233100, China
[b] College of Animal Science and Technology, Tarim University, Alae, 843300, China
[c] Anhui Province Key Laboratory of Animal Nutritional Regulation and Health, Anhui Science and Technology University, Fengyang, 233100, China
[d] School of Finance and Economics, Anhui Science and Technology University, Bengbu, 233030, China

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ABSTRACT

This study aimed to determine the effects of tributyrin on growth performance, gastrointestinal tract development, ruminal bacteria and volatile fatty acid (VFA) formation. Thirty healthy weaned Small-Tailed Han female lambs at 3 months old with BW 27.5 ± 4.1 kg (mean ± SD) were randomly assigned to five groups of six lambs each, and each group received tributyrin at 0, 0.5, 1.0, 2.0 and 4.0 g/kg in feed. Weights were measured before the start and end of the study. After 15 d adaptation, DMI, feed, faeces and urine were recorded every week. Lambs were sacrificed at d 75. Compared to lambs fed no tributyrin, lambs fed 4.0 g/kg tributyrin had higher average daily BW gain ($P = 0.04$) and DMI ($P < 0.01$). Tributyrin reduced nitrogen ($P < 0.01$), Ca ($P < 0.01$) and P ($P < 0.01$) losses derived from faeces and urine. The mostly important, tributyrin increased dorsal sac thickness ($P < 0.01$), papillae length ($P = 0.04$) and width ($P < 0.01$), ventral sac papillae length ($P < 0.01$) and width ($P < 0.01$), caudodorsal blind sac thickness ($P = 0.02$), papillae length ($P < 0.01$) and width ($P < 0.01$). Furthermore, tributyrin increased thicknesses of both the duodenum ($P < 0.01$) and ileum ($P = 0.01$), and villus heights of the duodenum ($P = 0.01$), ileum ($P < 0.01$), jejunum ($P < 0.01$) and caecum ($P = 0.02$), but tributyrin decreased duodenal ($P < 0.01$) and caecal crypt depths ($P < 0.01$). Tributyrin reduced rumen pH ($P < 0.01$) while promoting total VFA concentration ($P < 0.01$). Tributyrin improved the structure of rumen bacteria by enhancing *Clostridium* ($P = 0.04$), *Butyrivibrio* ($P < 0.01$), *Streptococcus* ($P = 0.04$), *Prevotella* ($P = 0.04$), *Ruminobacter* ($P = 0.02$) and *Fibrobacter* ($P = 0.03$). In conclusion, tributyrin could stimulate gastrointestinal tract development by enhancing colonization of rumen VFA-producing bacteria, and dietary supplementation of tributyrin at 4.0 g/kg of DM was recommended for the weaned lambs.

1. Introduction

Butyric acid is an important rumen fermentation product, with a changed concentration from 0.002 to 17.3 mol/L in the rumen of weaning calves from birth to weaning (Niwińska et al., 2017). Butyric acid has an obviously important function in stimulating rumen development because it could serve as a key energy source for rumen epithelial cells and stimulate gene expression in epithelial cell proliferation and ultimately rumen maturation (Donohoe et al., 2012). Besides, butyric acid may also promote rumen mucosa development by reducing apoptosis (Mentschel et al., 2001). Górka et al. (2018a) evaluated potential of exogenous butyrate supplementation on stomach functions of sheep and

* Corresponding authors.
E-mail addresses: Renqc@ahstu.edu.cn (Q. C. Ren), Xuanjj@ahstu.edu.cn (J. J. Xuan).
[1] These authors contributed equally to this study.
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found that the exogenous butyrate could well improve rumen, omasum and abomasum development. Despite these multiple benefits suggesting that butyric acid has a good potential to be widely added into the diet to improve both health and performance of ruminant animals, its disadvantages of having a very short half-life in plasma, volatility and odor result in a limited application in practice. In fact, butyric acid is usually added into diets in the form of sodium butyrate (Araujo et al., 2015).

Tributyrin is a derivative of butyric acid, and it could be metabolized into three free butyric acid molecules in the gastrointestinal tract (GIT) of animals. Compared with butyric acid and its salts, tributyrin is more stable and less odorous, and has more favorable pharmacokinetics (Miyoshi et al., 2011). In recent years, tributyrin has attracted much attention and been widely used as a feed additive in diets to improve health and performance of ruminant animals. For dairy cows, rumen-bypassed tributyrin was shown to relieve heat stress and to promote production performance by reducing lymphocyte response (Guo et al., 2021). In adult Small-Tailed Han sheep, dietary supplementation with tributyrin has been shown to improve rumen microbial growth and metabolites, thereby resulting in higher fibrolytic enzyme activity and dietary nutrient utilization (Ren et al., 2018a, 2018b, 2018c). For pre-weaned dairy calves, Liu et al. (2022) showed that tributyrin added into milk replacer could promote intestinal development via enhancing the intestinal barrier and suppressing the inflammatory response.

For young ruminant animals, the promotion of rumen development is particularly important since promoting rumen development has a significant impact on later growth and production performance. So far, various strategies including an increase of scratch factor, a promotion of volatile fatty acid (VFA) formation in the rumen, bacteria supplementation, changed feeding schedule and feed particle size, and addition of nutrient source have been used to speed up rumen development (Muscato et al., 2002; Baldwin et al., 2004; Lesmeister et al., 2004). Our in vitro and in vivo studies demonstrated that tributyrin supplementation could significantly promote VFA formation, particularly butyric acid (Ren et al., 2018a, 2018b, 2018c; Song et al., 2020). We hypothesized that tributyrin supplementation had a good potential to improve GIT, particularly ruminal development of lambs. Therefore, to fully evaluate this potential, the present experiment was conducted to determine the effects of tributyrin on growth performance, nutrient digestibility and loss, GIT development, ruminal bacteria and VFA formation of weaned Small-Tailed Han female lambs, and to provide a scientific basis for tributyrin utilization as a feed additive in the diet of young ruminants.

2. Materials and methods

2.1. Animal ethics statement

The animal care protocol involved in the current experiment was approved by the Animal Ethics Committee of Anhui Science and Technology University (protocol no. AECASTU-2023007). The present sampling procedures were applied according to Guideline no.398 on Ethical Treatment of Experimental Animals (2006) derived from the Ministry of Science and Technology, China. All efforts were made to minimize the lambs' suffering.

2.2. Lambs and treatments

The feeding period lasted from June to August. Thirty healthy weaned Small-Tailed Han female lambs at 3 months old with BW 27.5 ± 4.1 kg (mean ± SD) were randomly assigned to five groups of six lambs each. Each lamb was separately fed in a metabolic cage (1.5 m × 1.5 m) with a perforated wooden floor. During the experiment, lambs had ad libitum access to water and feed. The total mixed ration (TMR; Table 1) was separately offered to lambs at 07:00 and 19:00 every day. Based on DM of the formulated TMR, each group received tributyrin (Perstorp (Shanghai) Chemical Products Trading Co. Ltd, Shanghai, China) at 0, 0.5, 1.0, 2.0 and 4.0 g/kg in feed. The selected dosage of tributyrin was according to Ren et al. (2018a), who provided tributyrin to adult Small-Tailed Han ewes at varied levels from 0 to 8 g/kg of DM in feed.

Table 1
Ingredients and nutrient composition of the total mixed ration feed for weaned Small-Tailed Han female lambs (%, as DM basis).

Items	Content
Ingredients	
Maize	25.0
Soybean meal	11.0
Ensiled total corn stover	35.0
Peanut straw	20.0
Garlic by-products	5.0
Premix[1]	4.0
Nutrients	
Metabolizable energy[2], MJ/kg	12.3
Crude protein	18.1
Ether extract	3.1
NDF	37.3
ADF	24.2
Non-fibre carbohydrate[3]	34.1
Ash	7.4
Ca	0.7
Total P	0.4

NDF = neutral detergent fibre; ADF = acid detergent fibre.
[1] Per kilogram of premix contained 154.44 kIU of vitamin A, 94 kIU of vitamin D_3, 338.2 kIU of vitamin E, 120 mg of I, 280 mg of Cu, 2,240 mg of Fe, 1,740 mg of Mn, 1,370 mg of Zn, 60 mg of Se, 16.8 mg of Co, 50 mg of Lys and 50 mg of Met.
[2] Metabolizable energy was based on calculated values (NRC, 2001).
[3] Non-fibre carbohydrate (%, DM basis) = 100 - (neutral detergent fibre + crude protein + ether extract + ash).

2.3. Measurements of growth performance and nutrient utilization

Before the start and end of the current experiment, each lamb was separately weighed and the BW was recorded to calculate daily BW gain and ratio of feed to BW gain.

The current experiment lasted 75 d, consisting of 15 d of adaptation to the diet, followed by 60 d of the experimental period. Dry matter intake and total excretion of both faeces and urine of each lamb were weekly recorded in the seventh day during the experimental period. After recording, feed, faeces and urine samples of each lamb were respectively collected according to the method of Ren et al. (2018a) for later nutrient analysis such as nitrogen (N), ash, Ca and P, neutral detergent fibre (NDF) and acid detergent fibre (ADF).

2.4. Slaughter and sample collection

At end of the experiment, lambs were sacrificed 3 h after morning feeding, and the slaughtering procedure was strictly carried out according to Slaughter Processing of MARA (2019). Immediately after slaughtering, organs such as heart, liver, spleen, lung, kidney and gallbladder were spread on polystyrene plates to measure their weights and to calculate the organ index as follows: Organ index (kg/100 kg live BW) = 100 × [organ (kg)/live BW (kg)]. In the present experiment, the carcass was measured and defined as the weight of the lamb's slaughtered body without head, lower limbs, all internal organs and fur after being left for 30 min.

Forestomach compartments including rumen, reticulorumen, omasum and abomasum were respectively removed and emptied from their digesta and then weighed. Rumen digesta was emptied

into the designated clean plastic basin, and pH values were measured using a portable meter (HI8424, Beijing Hanna Instruments Science & Technology Co. Ltd, Beijing, China) from different compartments. After pH determination, the rumen digesta were hand-mixed thoroughly, and about 200 g of the digesta was collected and stored at −80 °C until extraction of bacterial DNA, while another 500 g rumen digesta was manually collected and filtered using four layers of muslin to obtain ruminal aliquots. The obtained aliquots were centrifuged at 3,000 × *g* at 4 °C for 30 min. The supernatant of the centrifuged aliquots was collected and stored at −20 °C for later analysis of VFA concentration.

Four pieces of the whole-tissue (each size of 2 cm × 2 cm) from the rumen dorsal sac, ventral sac, caudodorsal blind sac and caudoventral blind sac were separately taken, and rinsed in 0.9% stroke-physiological saline solutions (Huaian Kelun, Huaian, Jiangsu province, China) until clear, then the pieces were stored in designated 10% formalin neutral fixative solutions (Nanchang Yulu, Nanchang, Jiangxi province, China). Meanwhile, the small intestine including the duodenum, jejunum and ileum, and large intestine including colon, caecum and rectum were spread on polystyrene plates to measure their lengths and weights. Pieces with length of 2 cm from the duodenum, jejunum, ileum and caecum were respectively taken, washed clearly using the 0.9% stroke-physiological saline solution and fixed with 10% formalin solution for later determination of intestinal development.

2.5. Chemical analysis

According to AOAC (2012), standard methods were selected to analyze the content of DM (930.15), N (984.13), ash (975.03), Ca (968.08) and P (965.17) in feed, faeces and urine. For N analysis, freeze-dried samples of both feed and faeces were prepared instead of drying them with hot temperature (e.g. 105 °C) to avoid underestimating N content. According to the methods of Van Soest et al. (1991), contents of both NDF and ADF were separately assayed with α-thermoamylase (Wuhan Xinxin Jiali Biotechnology Co. Ltd, Wuhan, Hubei province, China). According to NRC (2001), metabolizable energy of the feed was estimated.

2.6. Histological analysis

In the present study, histological analysis of the rumen was carried out according to the method of Górka et al. (2018a). The rumen samples were firstly dehydrated using an ethanol series, followed by clearing with xylene and embedding in paraffin. Before embedding, each rumen sample was divided into a 5- to 10-mm thick piece, then the piece was embedded in a separate paraffin block. Six-micrometer thick sections were cut from each paraffin block and stained using both hematoxylin and eosin for morphometry. During the determination, one square centimeter of the rumen sample was used to analyze length, width and density of rumen papillae and thickness of the rumen muscle. With the help of a stereoscopic microscope (version ZEISS SteREO Discovery V12, ZEISS, Jena, Germany) and AxioVision software (Zeiss), rumen papillae were cut off from the base to measure papilla length and width. Papilla width was measured at the middle point of the papilla.

Histological analysis of the intestine was carried out according to the method reported by Ren et al. (2020). The fixed intestine samples were removed from the 10% formalin solution and separately embedded in paraffin blocks. For each block, 3 to 4 mm thick sections were sectioned by LEICA RM2235 microtome (Leica Microsystems, Milton Keynes, UK) and stained with hematoxylin and eosin. Four microscopic fields per sample were selected to measure the intestinal muscle thickness, villus height and crypt depth using a Carl Zeiss Axioskop microscope (Carl Zeiss GmbH, Jena, Germany). In the present experiment, villus height was defined as the height from the villus tip to the villus crypt junction, while crypt depth was defined as invagination depth between adjacent villi.

2.7. Determination of VFA concentration

According to the method of Ren et al. (2018a), rumen VFA concentration was analyzed using a GC522 gas chromatograph (Wufeng instruments, Shanghai, China). Before analysis, 1 mL of rumen fluid was mixed with 0.3 mL of meta-phosphoric acid (25%, wt/vol) and left for 30 min, then the mixture was centrifuged at 15,000 × *g* at 4 °C for 10 min. Following centrifugation, the supernatant was filtered through 0.22 μm organic membrane filter (Jiete Bio-filtration, Guangzhou, Guangdong province, China) to determine the concentration of VFA. During determination, the temperature of the injector oven was set at 250 °C, the column oven at 120 °C and detector at 250 °C, respectively. In the current determination, caproic acid purchased from Sigma Aldrich (St Louis, MO) was selected as an internal standard.

2.8. The high-throughput sequencing analysis

In the present experiment, TRIzol agent and a Power Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) were used to extract rumen bacterial DNA, and both the concentration and quality of the DNA were detected using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). According to the report of Li et al. (2022), the 16S rRNA with primers of F: 5′-ACTCCTACGGGAGGCAGCA-3′ and R: 5′-GGACTACHVGGGTWTCTAAT-3′ were selected to identify the bacterial taxa. Then, 10 μmol/L primer, 10 μL buffer, 10 μL High GC enhancer, 1 μL dNTP, and 0.2 μL Q5 High-fidelity DNA polymerase and 60 ng genomic DNA were prepared and added into a total volume of 50 μL reaction mixture. The PCR reactions were set under the following thermal cycling conditions: initial denaturation for 5 min at 95 °C, followed by 30 cycles for 1 min at 95 °C, 1 min at 60 °C, 1 min at 72 °C, and a final extension for 10 min at 72 °C. All PCR runs, negative controls, samples and standards were run in triplicate. In the present experiment, Quant-iT dsDNA HS Reagent was used to quantify and pool the PCR products.

An Illumina Hiseq 2500 platform (Annoroad, Shanghai, China) was used to perform the high-throughput sequencing analysis of the digesta bacteria rRNA genes. Chimeric sequences were detected using UCHIME algorithm (UCHIME Algorithm), while useful tags were sequenced using Uparse software (Uparse v 7.0.1001) to compare with the reference database. The representative sequence was selected and screened for further annotation if a sequence was higher than 97%. The taxonomic information was annotated using Green Gene Database, while the abundances of the OTUs were normalized using a standard sequence number.

2.9. Calculation and statistical analysis

Apparent digestibility was calculated as follows:

Apparent digestibility (%) = 100 × [DMI (g/d) × dietary nutrient content (g/g) − daily faeces excretion (g/d) × faecal nutrient content (g/g) − daily urine excretion (mL/d) × urinary nutrient content (g/mL)]/[DMI (g/d) × dietary nutrient content (g/g)].

In the present study, losses of dietary nutrients, including Ca and P (%, DM basis), were estimated without effects derived from water. Dietary nutrient loss was calculated as follows:

Dietary nutrient loss (%) = 100 × [daily faeces excretion (g/d) × faecal nutrient content (g/g) + daily urine excretion (mL/d) × urinary nutrient content (g/mL)]/[DMI (g/d) × dietary nutrient content (g/g)].

SAS 9.4 (Statistical Analysis for Windows, SAS Institute Inc., Cary, NC) with PROC MIXED model were used to analyze the experimental data. Beneficial effects of dietary tributyrin supplementation were evaluated with Contrast (Control vs. tributyrin treatments), Linear and Quadratic effects. Meanwhile, the significance level of the comparison among tributyrin treatments mean was conducted using Duncan's multiple range test. In the present experiment, the difference between tributyrin treatment means was considered highly significant at $P < 0.01$ and significant at $0.01 \leq P < 0.05$, while $0.05 \leq P < 0.10$ was considered a tendency. The PROC MIXED model was used including random and fixed effects as follows: $Y_{ij} = \mu + L_i + T_j + \varepsilon_{ij}$, where Y_{ij} is the dependent variable, μ is the overall mean, L_i is the random effects of lambs ($i = 6$), T_j is the fixed effect of tributyrin supplements ($j = 0$, 0.5, 1.0, 2.0 and 4.0 g/kg), and ε_{ij} is the error term.

3. Results

3.1. Effects of tributyrin on growth and slaughter performances of Small-Tailed Han lambs

Compared to the lambs fed the diet without tributyrin, average daily BW gain of lambs fed tributyrin were linearly increased by 36.3%, 39.2%, 40.9% and 66.3% ($P = 0.03$). The daily DMI of lambs fed tributyrin linearly increased by 5.7%, 9.2%, 10.3% and 19.5% ($P < 0.01$), whereas the ratio of feed to BW gain of the lambs provided with tributyrin linearly decreased by 19.4%, 21.6%, 22.4% and 28.4% ($P = 0.01$). As shown in Table 2, there were no significant differences observed in both carcass and slaughter ratio.

3.2. Effect of tributyrin on dietary nutrient digestibility and loss of Small-Tailed Han lambs

Compared to the lambs without tributyrin supplementation, lambs fed tributyrin had higher daily intakes of N ($P < 0.01$), NDF ($P < 0.01$), ADF ($P < 0.01$), Ca ($P < 0.01$) and P ($P < 0.01$). As shown in Table 3, lambs fed tributyrin had lower losses of faecal N ($P < 0.01$) and urinary N ($P < 0.01$), faecal Ca ($P = 0.01$) and urinary Ca ($P < 0.01$), faecal P ($P < 0.01$) and urinary P ($P < 0.01$) but higher digestibility of both NDF ($P < 0.01$) and ADF ($P = 0.03$) compared with that of lambs fed no tributyrin.

3.3. Effect of tributyrin on organ index of Small-Tailed Han lambs

As shown in Table 4, supplementing tributyrin had a positive effect on organ index, and lambs fed the diet with 2.0 g/kg tributyrin had a greater forestomach index ($P = 0.02$), rumen index

Table 2
Effects of tributyrin on growth and slaughter performances of weaned Small-Tailed Han female lambs.

Items	Tributyrin additions, g/kg DM basis					SEM	*P*-values[1]		
	0	0.5	1.0	2.0	4.0		Contrast	Linear	Quadratic
Daily BW gain, g/d	65.0^{b}	88.6^{ab}	90.5^{ab}	91.6^{ab}	108.1^{a}	12.28	0.04	0.03	0.96
DMI, kg/d	0.87^{c}	0.92^{bc}	0.95^{b}	0.96^{b}	1.04^{a}	0.022	<0.01	<0.01	0.64
Ratio of feed to BW, kg/kg	13.4^{a}	10.8^{ab}	10.5^{ab}	10.4^{ab}	9.6^{b}	1.24	0.02	0.01	0.56
Carcass, kg	13.7	14.5	14.7	14.5	15.4	0.99	0.35	0.30	0.90
Slaughter ratio, %	44.3	44.3	44.3	44.3	44.8	0.51	0.58	0.09	0.59

[a-c] Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1] Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.

Table 3
Effect of tributyrin on dietary nutrient digestibility and losses in weaned Small-Tailed Han female lambs.

Items	Tributyrin additions, g/kg DM basis					SEM	*P*-values[1]		
	0	0.5	1.0	2.0	4.0		Contrast	Linear	Quadratic
N intake, g/d	25.5^{c}	27.2^{bc}	28.1^{b}	28.2^{b}	30.7^{a}	0.69	<0.01	<0.01	0.56
Faecal N, g/d	14.0^{a}	12.1^{b}	12.7^{ab}	12.8^{ab}	12.5^{b}	0.44	<0.01	0.11	0.40
Urinary N, g/d	1.10^{a}	0.85^{b}	0.79^{b}	0.65^{b}	0.80^{b}	0.077	<0.01	<0.01	0.33
N loss, %	59.2^{a}	48.4^{b}	49.7^{b}	50.0^{b}	44.4^{b}	1.92	<0.01	<0.01	0.61
Faecal N loss, %	54.8^{a}	45.2^{bc}	46.9^{bc}	47.6^{b}	41.7^{c}	1.87	<0.01	<0.01	0.68
Urinary N loss, %	4.4^{a}	3.2^{b}	2.8^{b}	2.4^{b}	2.7^{b}	0.28	<0.01	<0.01	0.48
NDF intake, g/d	324^{b}	345^{ab}	357^{a}	358^{a}	389^{a}	8.1	<0.01	<0.01	0.54
Faecal NDF, g/d	240	235	232	228	217	9.6	0.24	0.07	0.91
NDF digestibility, %	26.2^{c}	32.4^{bc}	33.3^{ab}	35.9^{ab}	43.2^{a}	2.46	<0.01	<0.01	0.85
ADF intake, g/d	211^{c}	225^{bc}	232^{b}	232^{b}	252^{a}	5.6	<0.01	<0.01	0.57
Faecal ADF, g/d	162	169	178	166	163	7.2	0.37	0.95	0.36
ADF digestibility, %	23.7^{b}	25.5^{b}	21.4^{b}	29.1^{ab}	34.9^{a}	2.69	0.03	<0.01	0.17
Daily Ca intake, g/d	6.1^{c}	6.5^{bc}	6.8^{b}	6.7^{b}	7.4^{a}	0.20	<0.01	<0.01	0.52
Faecal Ca, mg/d	485	462	455	460	485	28.8	0.83	0.90	0.97
Urinary Ca, g/d	3.4	3.4	3.0	3.2	3.4	0.15	0.25	0.54	0.27
Dietary Ca loss, %	65.3^{a}	59.2^{ab}	52.4^{b}	55.6^{b}	53.5^{b}	2.44	<0.01	<0.01	0.20
Faecal Ca loss, %	8.1^{a}	7.0^{ab}	6.9^{ab}	7.0^{ab}	6.7^{b}	0.42	0.01	0.04	0.87
Urinary Ca loss, %	57.2^{a}	52.2^{ab}	45.5^{b}	48.6^{b}	46.8^{b}	2.32	<0.01	<0.01	0.17
Daily P intake, g/d	3.0^{c}	3.2^{bc}	3.4^{b}	3.4^{b}	3.7^{a}	0.08	<0.01	<0.01	0.52
Faecal P, mg/d	551^{a}	553^{a}	440^{b}	366^{b}	369^{b}	34.1	<0.01	<0.01	0.68
Urinary P, mg/d	49.9^{a}	43.7^{ab}	30.4^{c}	35.4^{bc}	39.0^{bc}	3.22	<0.01	<0.01	0.11
Dietary P loss, %	19.4^{a}	18.4^{a}	14.1^{b}	12.0^{bc}	11.1^{c}	0.85	<0.01	<0.01	0.35
Faecal P loss, %	17.6^{a}	17.0^{a}	13.1^{b}	10.9^{bc}	10.0^{c}	0.86	<0.01	<0.01	0.48
Urinary P loss, %	1.8^{a}	1.4^{b}	0.9^{c}	1.1^{bc}	1.1^{bc}	0.11	<0.01	<0.01	0.11

NDF = neutral detergent fibre; ADF = acid detergent fibre.
[a-c] Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1] Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.

Table 4
Effect of tributyrin on organ index of weaned Small-Tailed Han female lambs (% BW)[1].

Items	Tributyrin additions, g/kg DM basis					SEM	*P*-values[2]		
	0	0.5	1.0	2.0	4.0		Contrast	Linear	Quadratic
Forestomach[3]	2.67^{b}	3.29^{a}	2.89^{ab}	3.36^{a}	3.27^{a}	0.175	0.02	0.04	0.04
Rumen	1.63^{b}	1.97^{ab}	1.81^{ab}	2.08^{a}	1.99^{a}	0.105	0.01	0.03	0.07
Reticulum	0.27^{c}	0.33^{ab}	0.29^{bc}	0.36^{a}	0.32^{abc}	0.018	0.02	0.06	0.02
Omasum	0.37	0.52	0.38	0.43	0.45	0.059	0.30	0.71	0.17
Abomasum	0.38	0.45	0.40	0.47	0.49	0.047	0.19	0.12	0.30
Heart	0.34	0.32	0.32	0.34	0.34	0.018	0.39	0.80	0.95
Liver	1.19	1.26	1.12	1.27	1.32	0.086	0.61	0.34	0.22
Spleen	0.12	0.13	0.13	0.13	0.15	0.010	0.42	0.15	0.49
Lung	1.75	1.63	1.71	1.93	1.72	0.138	0.99	0.57	0.64
Kidney	0.25	0.23	0.24	0.29	0.23	0.021	0.93	0.74	0.33
Gallbladder	0.03	0.02	0.03	0.03	0.03	0.009	0.90	0.44	0.29
Intestine[4]	2.82	3.08	3.04	3.07	3.06	0.180	0.26	0.45	0.73
Small intestine	1.51	1.75	1.91	1.66	1.82	0.129	0.08	0.20	0.30
Duodenum	0.06	0.15	0.14	0.06	0.10	0.033	0.19	0.91	0.52
Jejunum	1.28	1.32	1.34	1.45	1.52	0.151	0.46	0.23	0.86
Ileum	0.16	0.27	0.42	0.15	0.20	0.079	0.27	0.89	0.09
Large intestine	1.31	1.33	1.13	1.41	1.23	0.096	0.68	0.73	0.06
Colon	0.57	0.60	0.48	0.57	0.41	0.053	0.40	0.06	0.08
Caecum	0.39	0.39	0.30	0.38	0.51	0.059	0.99	0.26	0.43
Rectum	0.34	0.33	0.34	0.44	0.30	0.044	0.89	0.89	0.27

[a-c] Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1] Organ index was calculated as follows: Organ index (%) = [organ (kg)/live BW (kg)] ×100.
[2] Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.
[3] Forestomach including the rumen, reticulum, omasum and abomasum.
[4] Intestine including the duodenum, jejunum, ileum, colon, caecum and rectum.

($P = 0.01$) and reticulum index ($P = 0.02$) than that of the lambs fed the basal diet only. In the present study, there were no significant effects of tributyrin on the indices of other organs such as the omasum, abomasum, heart, liver, spleen, lung, kidney, gallbladder and large intestine, despite tributyrin having a tendency to increase the small intestine index ($P = 0.08$).

3.4. Effect of dietary supplementation with tributyrin on rumen development

As shown in Table 5, compared to the lambs fed a basal diet only, dorsal sac thicknesses of lambs fed the diet with tributyrin linearly increased by 3.6%, 24.9%, 34.3% and 20.8% ($P < 0.01$). In addition, dietary supplementation with tributyrin linearly increased both papillae length ($P = 0.03$) and width ($P < 0.01$) of the dorsal sac. Ventral sac papillae length ($P = 0.02$) and width ($P = 0.03$) quadratically increased with increasing tributyrin. Lambs fed the diet with 2.0 g/kg tributyrin had greater caudodorsal blind sac thickness ($P = 0.02$) and papillae length ($P < 0.01$) than that of the lambs fed basal diet only, while the lambs fed the diet with 4.0 g/kg tributyrin had higher caudodorsal blind sac papillae width ($P < 0.01$). In the present study, there were no significant differences observed in caudoventral blind sac thickness, papillae density, length and width.

Table 5
Effect of tributyrin on rumen development in weaned Small-Tailed Han female lambs.

Items	Tributyrin additions, g/kg DM basis					SEM	*P*-values[1]		
	0	0.5	1.0	2.0	4.0		Contrast	Linear	Quadratic
Thickness, μm									
Dorsal sac	1,565^{b}	1,622^{b}	1,955^{a}	2,103^{a}	1,891^{a}	86.6	<0.01	<0.01	0.72
Ventral sac	1,571	1,575	1,744	1,599	1,671	77.0	0.33	0.33	0.16
Caudodorsal blind sac	1,315^{b}	1,332^{b}	1,574^{ab}	1,728^{a}	1,497^{ab}	92.5	0.02	<0.01	0.98
Caudoventral blind sac	1,529	1,511	1,599	1,876	1,613	134.6	0.29	0.11	0.45
Papillae density, /cm^2									
Dorsal sac	111	109	99	92	94	9.5	0.11	0.36	0.30
Ventral sac	109	107	111	103	106	9.3	0.90	0.21	0.35
Caudodorsal blind sac	108	102	124	107	102	8.1	0.85	0.41	0.86
Caudoventral blind sac	106	110	113	103	102	7.6	0.05	0.19	0.86
Papillae length, μm									
Dorsal sac	1,151^{b}	1,373^{ab}	1,344^{ab}	1,425^{ab}	1,545^{a}	127.9	0.04	0.03	0.71
Ventral sac	1,153^{b}	1,845^{a}	1,529^{ab}	1,469^{ab}	1,429^{b}	128.2	<0.01	0.64	0.02
Caudodorsal blind sac	945^{c}	1,377^{ab}	1,186^{abc}	1,439^{a}	1,092^{bc}	109.3	<0.01	0.28	0.04
Caudoventral blind sac	1,183	1,299	1,502	1,501	1,364	161.2	0.08	0.16	0.77
Papillae width, μm									
Dorsal sac	378^{c}	402^{bc}	441^{bc}	512^{a}	464^{ab}	21.1	<0.01	<0.01	0.41
Ventral sac	392^{b}	535^{a}	466^{ab}	475^{ab}	404^{b}	28.1	<0.01	0.65	0.03
Caudodorsal blind sac	350^{c}	362^{c}	383^{bc}	437^{ab}	476^{a}	21.9	<0.01	<0.01	0.72
Caudoventral blind sac	402	390	444	416	470	33.6	0.32	0.06	0.24

[a-c] Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1] Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.

3.5. Effect of dietary supplementation with tributyrin on intestine development

As shown in Table 6, there were no significant differences observed in the lengths of total intestine, small intestine including both jejunum and ileum and large intestine including colon, caecum and rectum. However, lambs fed the diet with 2.0 g/kg tributyrin had greater length of duodenum ($P = 0.04$). In the present study, lambs fed the diet with 1.0 g/kg tributyrin had higher duodenum thickness ($P < 0.01$) and ileum thickness ($P = 0.01$). Compared to lambs fed the basal diet only, lambs fed tributyrin supplementation had greater villus height in the duodenum ($P = 0.01$), jejunum ($P < 0.01$), ileum ($P < 0.01$) and caecum ($P = 0.02$) but lower crypt depth in both the duodenum ($P < 0.01$) and caecum ($P < 0.01$).

3.6. Effects of tributyrin on rumen pH value and VFA formation

Compared to lambs fed the basal diet only, rumen pH values of the lambs fed the diet with tributyrin supplementation linearly decreased by 2.3%, 4.2%, 7.1% and 3.3% ($P < 0.01$), while rumen total VFA concentration linearly increased by 6.9%, 10.4%, 20.1% and 12.0% ($P < 0.01$). As shown in Table 7, supplementing tributyrin increased molar proportions of acetic acid ($P = 0.02$), valeric acid ($P < 0.01$) and branched-chain VFA ($P < 0.01$) while decreasing propionic acid ($P < 0.01$). In the present study, the molar proportion of butyric acid was not significantly different among tributyrin treatments.

3.7. Effect of tributyrin on the relative abundance of rumen digesta bacteria

At phylum level, supplementing tributyrin increased the relative abundances of Firmicutes ($P < 0.01$), Bacteroidetes ($P = 0.01$) and Fibrobacteres ($P = 0.03$), and tributyrin tended to increase the relative abundance of Proteobacteria ($P = 0.05$), but tributyrin had no significant effects on both Spirochaetes and Actinobacteria (as shown in Table 8). At genus level, supplementing tributyrin enhanced the relative abundances of *Clostridium* ($P = 0.04$), *Butyrivibrio* ($P < 0.01$), *Streptococcus* ($P = 0.04$), *Prevotella* ($P = 0.04$), *Ruminobacter* ($P = 0.02$) and *Fibrobacter* ($P = 0.03$), but tributyrin had no significant effects on *Lactobacillus*, *Bacteroides*, *Parabacteroides* and *Bifidobacterium*.

4. Discussion

Limited research determining the effects of tributyrin on weaned lambs is currently available. The present study showed that supplementing tributyrin affected the growth performance, rumen and intestine development, digesta bacteria colonization and VFA concentration in the rumen of weaned Small-Tailed Han female lambs.

4.1. Beneficial effect of tributyrin on growth performance of weaned lambs

In the present study, supplementing tributyrin to weaned lambs has been shown to improve growth performance by increasing average daily weight gain and feed efficiency, possibly due to the stimulatory effect of tributyrin on GIT development, including the rumen and small intestine. It is well known that the GIT of ruminants presents a uniquely organized system, and GIT development can directly affect intake of feed, nutrient digestibility and overall growth (Diao et al., 2019). Thereby improving rumen and intestine development can lead to an improved growth performance of lambs fed a diet with tributyrin supplementation. The obtained results were consistent with the findings of Murayama et al. (2023), who showed that tributyrin supplementation in milk replacer

Table 6
Effect of tributyrin on intestinal development in weaned Small-Tailed Han female lambs.

Items	Tributyrin additions, g/kg DM basis					SEM	*P*-values[1]		
	0	0.5	1.0	2.0	4.0		Contrast	Linear	Quadratic
Intestine length, cm									
Total intestine[2]	5,092	5,521	5,445	5,879	5,368	413.6	0.34	0.50	0.49
Small intestine	2,276	2,441	2,451	2,643	2,398	188.4	0.34	0.47	0.55
Duodenum	70^{b}	120^{ab}	135^{ab}	150^{a}	140^{ab}	26.9	0.04	0.02	0.78
Jejunum length	2,073	2,084	2,074	2,316	2,057	189.1	0.78	0.74	0.53
Ileum length	133	236	242	176	200	45.8	0.14	0.62	0.73
Large intestine length	540	639	542	592	572	55.7	0.47	0.92	0.25
Colon length	370	425	333	286	296	47.7	0.52	0.08	0.66
Caecum length	74	103	122	164	183	50.7	0.24	0.11	0.84
Rectum length	95	110	88	142	93	34.0	0.75	0.81	0.32
Small intestine to total intestine ratio, cm/cm	0.44	0.44	0.45	0.44	0.44	0.003	0.92	0.48	0.37
Intestine thickness, μm									
Duodenum	102^{c}	120^{b}	137^{a}	117^{bc}	115^{bc}	5.1	<0.01	0.20	0.05
Jejunum	103	119	112	108	101	7.6	0.40	0.48	0.63
Ileum	79^{b}	98^{ab}	115^{a}	96^{ab}	82^{b}	6.6	0.01	0.88	0.02
Caecum	230	289	272	252	312	20.9	0.06	0.09	0.95
Villus height, μm									
Duodenum	135^{b}	137^{b}	152^{ab}	157^{a}	159^{a}	5.5	0.01	<0.01	0.54
Jejunum	138^{c}	182^{ab}	201^{a}	164^{b}	165^{b}	8.4	<0.01	0.20	0.03
Ileum	142^{c}	165^{b}	150^{bc}	151^{bc}	183^{a}	5.3	<0.01	<0.01	0.43
Caecum	45^{b}	53^{a}	49^{ab}	55^{a}	50^{ab}	2.1	0.02	0.18	0.03
Crypt depth, μm									
Duodenum	172^{a}	144^{ab}	128^{b}	134^{b}	130^{b}	11.8	<0.01	0.01	0.72
Jejunum	218	233	168	177	205	27.3	0.47	0.34	0.39
Ileum	140	122	114	142	128	7.7	0.14	0.89	0.15
Caecum	144^{a}	121^{b}	120^{b}	85^{c}	110^{b}	6.5	<0.01	<0.01	0.02

$^{a-c}$ Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1] Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.
[2] Total length of intestine including both small and large intestines.

Table 7
Effects of tributyrin on rumen pH value and VFA concentration of weaned Small-Tailed Han female lambs.

Items	Tributyrin additions, g/kg DM basis					SEM	*P*-values[1]		
	0	0.5	1.0	2.0	4.0		Contrast	Linear	Quadratic
pH	7.28^{a}	7.11^{ab}	6.97^{b}	6.76^{c}	7.04^{b}	0.069	<0.01	<0.01	0.28
TVFA[2], mol/L	68.15^{d}	72.82^{c}	75.23^{bc}	81.85^{a}	76.33^{b}	0.908	<0.01	<0.01	<0.01
Acetic acid, mol/100 mol	73.81^{b}	74.59^{ab}	74.10^{b}	75.47^{a}	74.63^{ab}	0.317	0.02	0.02	0.01
Propionic acid, mol/100 mol	16.57^{a}	15.55^{b}	15.54^{b}	14.48^{c}	15.41^{b}	0.232	<0.01	<0.01	0.01
Butyric acid, mol/100 mol	6.65	6.63	6.88	6.73	6.71	0.171	0.64	0.69	0.41
Valeric acid, mol/100 mol	1.29^{c}	1.43^{b}	1.57^{a}	1.38^{bc}	1.35^{bc}	0.034	<0.01	0.61	<0.01
BCVFA[3], mol/100 mol	1.65^{b}	1.77^{ab}	1.88^{a}	1.91^{a}	1.87^{a}	0.048	<0.01	<0.01	0.80

TVFA = total volatile fatty acid; BCVFA = branched-chain VFA.
$^{a-d}$ Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1] Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.
[2] TVFA including acetic acid, propionic acid, butyric acid, valeric acid and BCVFA.
[3] BCVFA including iso-butyric acid and iso-valeric acid.

Table 8
Effects of tributyrin on the relative abundance of rumen digesta bacteria in weaned Small-Tailed Han female lambs.

Items	Tributyrin additions, g/kg DM basis					SEM	*P*-values[1]		
	0	0.5	1.0	2.0	4.0		Contrast	Linear	Quadratic
At phylum level									
Firmicutes	14.09^{b}	14.97^{b}	18.74^{a}	17.70^{a}	18.33^{a}	0.740	<0.01	<0.01	0.03
Bacteroidetes	31.19^{b}	32.30^{ab}	33.66^{a}	32.68^{ab}	32.56^{ab}	0.510	0.01	0.06	0.18
Proteobacteria	2.30	2.18	1.97	1.89	1.94	0.135	0.05	0.03	0.86
Spirochaetes	1.31	1.29	1.21	1.18	1.25	0.067	0.20	0.24	0.93
Actinobacteria	0.75	0.82	0.87	0.72	0.74	0.051	0.48	0.42	0.20
Fibrobacteres	0.47^{b}	0.52^{b}	0.59^{a}	0.53^{ab}	0.50^{b}	0.023	0.03	0.33	0.02
At genus level									
Clostridium	2.01^{b}	2.17^{ab}	2.60^{a}	2.34^{ab}	2.21^{ab}	0.157	0.04	0.25	0.02
Butyrivibrio	1.01^{b}	1.43^{ab}	1.65^{a}	1.60^{a}	1.54^{a}	0.168	<0.01	0.03	0.81
Lactobacillus	0.08	0.08	0.09	0.08	0.08	0.005	0.77	0.93	0.62
Streptococcus	0.06^{b}	0.06^{b}	0.07^{ab}	0.07^{ab}	0.09^{a}	0.004	0.04	<0.01	0.80
Prevotella	18.09^{b}	20.39^{ab}	19.65^{ab}	20.38^{ab}	21.67^{a}	1.058	0.04	0.04	0.54
Bacteroides	2.72	2.94	2.91	2.89	2.95	0.178	0.32	0.48	0.86
Parabacteroides	0.53	0.60	0.62	0.58	0.58	0.049	0.23	0.62	0.84
Ruminobacter	0.10^{b}	0.13^{ab}	0.12^{ab}	0.14^{a}	0.14^{a}	0.010	0.02	0.02	0.28
Bifidobacterium	0.09	0.10	0.11	0.11	0.12	0.012	0.10	0.09	0.98
Fibrobacter	0.79^{b}	1.20^{ab}	1.12^{ab}	1.56^{a}	1.68^{a}	0.236	0.03	<0.01	0.50

$^{a, b}$ Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1] Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.

could improve growth performance and gut health of Holstein dairy calves.

4.2. Effects of tributyrin on DMI, dietary nutrient digestibility and loss in weaned lambs

Recent years, tributyrin as a feed additive has been widely used in animals. The present study showed that tributyrin had a significantly positive effect on the DMI of lambs, and this may be due to the anti-stress functions of tributyrin. As described earlier, the present experiment was carried out between June and August, and the lambs were inevitably affected by heat stress during the experiment, despite all efforts being made to minimize the stress. Guo et al. (2021) showed that dietary supplementation with tributyrin could relieve heat stress in dairy cows by reducing the inflammatory responses of lymphocytes. Furthermore, Liu et al. (2021) demonstrated that tributyrin at the level of 2.0 g/L in pasteurized waste milk could alleviate oxidative stress and inflammatory status in dairy calves by reducing blood haptoglobin, endothelin and IL-1β concentrations. Due to the advantage of tributyrin on alleviating stress, it is believed that lambs fed a diet with tributyrin supplementation, particularly higher dosages, had greater DMI. The present results indicated that dietary supplementation with tributyrin could stimulate feed intake of weaned lambs, and this was in agreement with the observed result of Murayama et al. (2023), who reported that Holstein dairy calves fed tributyrin with a dosage of 6.0 g/kg DM basis had higher DMI during postweaning.

For ruminant animals, higher excretions of N and minerals such as Ca and P in faeces and urine not only pollute the environment but also result in feed resources wastage. Tan et al. (2021) reported that rumen microbial metabolism of dietary protein is an obviously important factor affecting N excretion through faeces and urine. It is well known that rumen microbial protein is an important source of metabolizable protein for ruminant animals due to its good metabolic quantity and excellent amino acid profile, thus there is good reason to expect that microbial growth efficiency is higher in low quality feed. Previous studies have shown that dietary supplementation with tributyrin could promote the yield of rumen microbial protein (Ren et al., 2018c), which may contribute to higher dietary N absorption in the intestine and lower daily excretion of N in both faeces and urine. Hoenderop et al. (2005) pointed out that the major absorption site of Ca and P mainly occurs in the small intestine, particularly the duodenum and upper jejunum in monogastric animals. Marked differences to monogastric animals exist in ruminants, such as Ca absorption occurring along the whole gastrointestinal axis, particularly in the rumen (Wilkens and Muscher-Banse, 2020). In the present study, dietary supplementation with tributyrin had beneficial effects on rumen and intestine development, which may contribute to Ca and P

absorption, thereby resulting in lower excretion of Ca and P in both faeces and urine. Tributyrin could enhance rumen microbial growth and fibrolytic enzymes such as xylanase, carboxymethyl cellulase and avicelase (Ren et al., 2018a), thus dietary supplementation with tributyrin may increase digestibility of dietary NDF and ADF. The present results were in agreement with Li et al. (2022), who reported that supplementing tributyrin could greatly increase the apparent digestibility of both dietary crude protein and NDF in weaned lambs.

4.3. Beneficial effect of tributyrin on rumen development

For ruminant animals, the rumen plays an obviously important role in nutrient fermentation and absorption. However, the rumen is incompletely developed both physically and metabolically in newborn ruminants. Following the initiation of solid feed intake and colonization of rumen microbiota, in particular the production of VFA, the rumen undergoes physical and metabolic maturation. The physical development of the rumen can be further partitioned into two aspects, including increases in both rumen mass and papillae growth (Baldwin et al., 2004). Generally, good rumen development is recognized as a substantial increase in the amount and size of rumen papillae.

Based on previous literature, it seems that additional acceleration of rumen development can be obtained by dietary supplementation with butyrate (Górka et al., 2018b). The present study showed that dietary supplementation with tributyrin could also effectively facilitate rumen development in lambs, and this may be due to the highly significant promoting effect of tributyrin on VFA formation in the rumen. To date, there are at least two important mechanisms for VFA to stimulate rumen development. On one hand, the stimulatory effect of butyrate on rumen epithelium growth has been known for years and its contribution of energy, by providing available energy source for rumen epithelia cells, has been considered as a specific molecular mechanism of butyrate to speed up rumen development (Niwińska et al., 2017). Recently, Sun et al. (2021), using transcriptomic analysis, revealed that VFA as an energy substance during its metabolism may directly stimulate rumen development, and both VFA absorption and metabolism are regulated via the peroxisome proliferator-activated receptor signaling pathway. On the other hand, VFA affects the expression of various genes in the ruminal epithelium. Lin et al. (2019) reported that the promotion of microbiome-driven generation of ruminal VFA such as acetate and butyrate could upregulate the relative expression of various growth-related genes in the ruminal epithelium such as *MAPK1, PIK3CB, TNFSF10, ITGA6, SNAI2, SAV1* and *DLG*, whereas VFA significantly downregulated the *BAD* gene, related to cell death. In addition, butyrate infusion into the rumen could promote rumen papillae growth by enhancing the expression of genes related to epithelial VFA uptake and metabolism in preweaning twin lambs (Liu et al., 2019). Based on previous and present results, it is believed that both butyrate and tributyrin could exert a similar stimulatory effect on rumen development, despite the lack of more available evidence.

4.4. Beneficial effect of tributyrin on small intestine development

The small intestine is an important tissue for animals, and better intestinal development may increase nutrient absorption (Mekbungwan et al., 2002) and growth performance (Swiatkiewicz and Hanczakowska, 2006). So far, little is known about the benefit of tributyrin added into solid feed on the development of the small intestine of ruminants. Some information about the effect of tributyrin on intestinal development can be extrapolated from studies in simple-stomached species. Accordingly, Chen et al. (2022) demonstrated that dietary supplementation with tributyrin could significantly increase the occludin expression level and ileum villus height in weaned piglets, and Li et al. (2016) showed the promoting effects of tributyrin on intestinal digestive and barrier functions in intrauterine growth-restricted piglets. However, possible species differences have to be taken into account.

In the present study, the beneficial effects of tributyrin supplementation on intestinal development of weaned lambs were observed, and this may be explained with two possible reasons. On one hand, dietary supplementation with tributyrin could increase DMI of lambs, thereby resulting in more available metabolic energy and nutrition to meet the demands of intestinal development, particularly the small intestine. Previous studies have shown that there is a positive relationship between energy intake level and gut mass change (Johnson et al., 1990; Burrin et al., 1992; Freetly et al., 1995). Lately, McLeod and Baldwin (2000) proved that increasing intakes of metabolic energy and dietary forage in sheep could result in an increase in intestinal growth via cellular hyperplasia. In fact, dietary supplementation with tributyrin not only resulted in higher dietary metabolic energy and forage intake for weaned lambs but also directly provided butyric acid by the action of intestinal lipase, which serves as a major energy source for gastrointestinal epithelial cells and promotes normal epithelial cell growth, particularly intestinal villi. On the other hand, tributyrin administration could improve the development and health of the intestine by stimulating colonization of VFA-producing bacteria, enhancing barrier function and suppressing inflammatory responses in dairy calves fed milk replacer (Liu et al., 2022). The latest study by Zhong et al. (2023) seems to support the findings of Liu et al. (2022) in that dietary butyrate exhibited a promoting effect on the jejunal development of calves fed a high fiber starter by inhibiting inflammation, enhancing immunity and activating microbial metabolism. To date, there have been limited studies investigating the inclusion of tributyrin into lamb diets, and more research is required to determine if a similar effect of butyrate observed in calves could be achieved in lambs fed with tributyrin.

4.5. Benefits of tributyrin on rumen bacterial colonization and VFA formation

A variety of microbes including bacteria, protozoa, fungi and archaea colonize the rumen and they ferment feed components such as carbohydrate and crude protein to produce metabolites such as VFA and microbial protein to meet growth and production requirements for both themselves and their hosts. Early studies reported that rumen microbes may supply 70% to 100% of amino acid requirements for ruminants and provide 70% to 85% of the absorbed energy (AFRC, 1992; Dewhurst et al., 1986). For these beneficial aspects, optimizing rumen microbial growth and metabolites is particularly important.

Among the rumen microbes, digesta bacteria play an obviously important role in the biological degradation of plant fiber due to their much larger biomass as well as higher activity (Koike and Kobayashi, 2009). In the present study, colonization of rumen digesta bacteria was affected by dietary supplementation with tributyrin, and greatly varied abundances at both phylum and genus levels were observed. Among the present detected rumen bacteria, Bacteroidetes and Firmicutes were the most numerous groups, followed by Proteobacteria, Spirochaetes, Actinobacteria and Fibrobacteres. At genus level, the results showed that dietary supplementation with tributyrin could enhance the relative abundances of *Prevotella*, *Butyrivibrio* and *Fibrobacter*, which have been recognized as obviously important fibrolytic bacteria (Koike and Kobayashi, 2009). Similar results were reported in an earlier study by Li et al. (2012), who illustrated that exogenous butyrate

infusion greatly enhanced the relative abundance of bacteria in the rumen of dairy cows, especially Firmicutes. Besides, Liu et al. (2022) showed that supplementing tributyrin could optimize intestine microbial growth, particularly VFA-producing bacteria such as *Prevotella*, Lachnospiraceae, Ruminococcaceae and Rikenellaceae. To date, little is known about the potential mechanism of how butyrate and tributyrin influence GIT microbiota and VFA-producing bacteria. It has long been known that VFA-producing bacteria play key roles in maintaining health, promoting organ and tissue development, and improving the growth and productivity of ruminants (Yeoman and White, 2014). For the above reasons, hosts usually do not generate colonization resistance for these bacteria. And, calves and lambs would help even enhance the bacteria colonization by inhibiting harmful bacteria or via other pathways. Recently, Zhang et al. (2023) reported that butyrate could effectively improve the intestine microbial structure in weanling rabbits by suppressing harmful bacteria and promoting beneficial ones. It is worth mentioning that so far not a single report is available that presents the effect of tributyrin supplementation on rumen microbial structure in ruminants. With transcriptomic analysis, our unpublished data showed that dietary supplementation with tributyrin could upregulate the relative expression of *BPIFA1* mRNA in the rumen, and the correlation between the relative expression and tributyrin dosage demonstrated that the relative expression of *BPIFA1* mRNA was higher in the rumen of the lambs fed the diet with tributyrin dosage of at least 1.0 g/kg DM basis. Interestingly, the relative expression of *BPIFA1* mRNA in the rumen was significantly correlated with the relative abundances of *Prevotella*, *Butyrivibrio*, *Streptococcus* and *Fibrobacter*, and this was consistent with Wheeler et al. (2011), who reported that the BPIFA1 protein is an important molecular regulator to enhance colonization of specific rumen microbiota such as fibrolytic bacteria. Since the colonization of rumen microbiota is a very complex process and is seriously affected by diet and host, more research is required to determine the advantages of tributyrin supplementation on the colonization of rumen microbiota.

Volatile fatty acids, also called short-chain fatty acids, including acetic acid, propionic acid, butyric acid, valeric acid and branched-chain VFA (iso-butyric acid and iso-valeric acid), are the major metabolites of rumen microbes. In the present study, VFA concentration in the rumen digesta was greatly promoted by supplementing tributyrin, which may be due to the stimulating effect of tributyrin on the rumen microbial population and metabolism, especially VFA-producing bacteria. For example, the present experiment demonstrated that supplementing tributyrin could enhance *Prevotella*, which is one of the most numerous groups of rumen bacteria and produces acetic acid, succinate and propionic acid (Hobson and Stewart, 1988). *Butyrivibrio*, a famous major butyric acid-producing bacteria (Guo and Li, 2019), experiences better growth through stimulation by tributyrin, which is beneficial for increasing butyric acid formation in the rumen. Among the fibrolytic bacteria, *Fibrobacter* has been considered the predominant fibrolytic bacteria in sheep and its fermentation products include acetic acid, succinate, formate, propionic acid and valeric acid (Hobson and Stewart, 1988), which may contribute to the promotion of VFA formation in the rumen when its population has been enhanced by tributyrin supplementation.

4.6. Recommended dose

In general, the recommended levels of butyrate supplementation in feed for lambs are low. Cavini et al. (2015) showed that butyrate supplementation at the level of 3.0 g/kg of DM was sufficient to elicit a stimulatory effect on GIT development, feed intake and growth performance in preweaning lambs, but the level was insufficient to sustain such an effect after weaning. In the present experiment, it has been shown that tributyrin supplementation with the dosage of 4.0 g/kg of DM could significantly exert positive effects on both GIT development and growth performance of weaned lambs, while a tributyrin dosage of 0.5 g/kg of DM was insufficient to significantly stimulate colonization of digesta bacteria and to obviously promote VFA formation in the rumen. Based on the stimulatory effect of tributyrin on growth performance, tributyrin supplementation at 4.0 g/kg of DM was recommended for 3-month-old weaned Small-Tailed Han female lambs.

5. Conclusions

It has been shown that dietary supplementation with tributyrin could enhance the colonization of ruminal bacteria, particularly VFA-producing bacteria such as *Prevotella*, *Clostridium*, *Butyrivibrio*, *Streptococcus*, *Ruminobacter* and *Fibrobacter*. The enhanced bacterial colonization resulted in a promotion of VFA formation in the rumen, which improved GIT development, particularly in the rumen and small intestine. Improved GIT development led to an improved DMI and reduced dietary nutrient excretion via faeces or urine. Based on the above stimulatory effects, supplementing tributyrin promoted the growth performance of weaned Small-Tailed Han female lambs. In the present study, the optimal tributyrin dosage of 4.0 g/kg of DM was recommended for the weaned lambs.

Author contributions

Zhiwei Li and **Xueer Wang:** Carrying out the experiment during the whole feeding period, Methodology, Data curation, Writing-Original Draft and Editing. **Wei Wang, Ran An,** and **Yaxin Wang**: Methodology, Investigation and Determination. **Qingchang Ren:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing, Reviewing and Editing. **Jingjing Xuan:** Conceptualization, Methodology, Investigation and Determination.

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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References

Agricultural and Food Research Council. Technical committee on response to nutrients No 9. Nutrients requirements of ruminant animal: protein. Nutr Abstr Rev (Series B) 1992;62:787–818.

AOAC. Official methods of analysis. 19th ed. Gaithersburg, MD: AOAC International; 2012.

Araujo G, Terré M, Mereu A, Ipharraguerre IR, Bach A. Effects of supplementing a milk replacer with sodium butyrate or tributyrin on performance and metabolism of Holstein calves. Anim Prod Sci 2015;56(11):1834–41.

Baldwin RL, McLeod KR, Klotz J, Heitmann RN. Rumen development, intestinal growth and hepatic metabolism in the pre-and postweaning ruminant. J Dairy Sci 2004;87:E55–65.

Burrin DG, Britton RA, Ferrell CL, Bauer ML. Level of nutrition and visceral organ protein synthetic capacity and nucleic acid content in sheep. J Anim Sci 1992;70:1137–45.

Cavini S, Iraira S, Siurana A, Foskolos A, Ferret A, Calsamiglia S. Effect of sodium butyrate administered in the concentrate on rumen development and productive performance of lambs in intensive production system during the suckling and the fattening periods. Small Rumin Res 2015;123:212–7.

Chen G, Zhuo R, Ding H, Yang K, Xue J, Zhang S, Chen LX, Yin YL, Fang R. Effects of dietary tributyrin and physterol ester supplementation on growth performance, intestinal morphology, microbiota and metabolites in weaned piglets. J Appl Microbiol 2022;132(3):2293–305.

Dewhurst RJ, Webster AJF, Wainman FW, Dewey PJS. Prediction of the true metabolizable energy concentration in forages for ruminants. Anim Sci 1986;43:183–94.

Diao QY, Zhang R, Fu T. Review of strategies to promote rumen development in calves. Animals 2019;9(8):490.

Donohoe DR, Collins LB, Wali A, Bigler R, Sun W, Bultman SJ. The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. Mol Cell 2012;48:612–26.

Freetly HC, Ferrell CL, Jenkins TG, Goetsch AL. Visceral oxygen consumption during chronic feed restriction and realimentation in sheep. J Anim Sci 1995;73:843–52.

Górka P, Śliwiński B, Flaga J, Olszewski J, Wojciechowski M, Krupa K, Godlewski MM, Zabielski R, Kowalski ZM. Effect of exogenous butyrate on the gastrointestinal tract of sheep. I. Structure and function of the rumen, omasum, and abomasum. J Anim Sci 2018a;96(12):5311–24.

Górka P, Kowalski ZM, Zabielski R, Guilloteau P. Invited review: use of butyrate to promote gastrointestinal tract development in calves. J Dairy Sci 2018b;101(6):4785–800.

Guo M, Li ZY. Polysaccharides isolated from *Nostoc commune* Vaucher inhibit colitis-associated colon tumorigenesis in mice and modulate gut microbiota. Food Funct 2019;10(10):6873–81.

Guo W, Liu J, Yang Y, Ma H, Gong Q, Kan XC, Ran X, Cao Y, Wang JF, Fu SP, Hu GQ. Rumen-bypassed tributyrin alleviates heat stress by reducing the inflammatory responses of immune cells. Poultry Sci 2021;100(1):348–56.

Hobson PN, Stewart CS, editors. Rumen microbial ecosystem. Springer Science & Business Media; 1988.

Hoenderop JG, Nilius B, Bindels RJ. Calcium absorption across epithelia. Physiol Rev 2005;85:373–422.

Johnson DE, Johnson KA, Baldwin RL. Changes in liver and gastrointestinal tract energy demands in response to physiological workload in ruminants. J Nutr 1990;120:649–55.

Koike S, Kobayashi Y. Fibrolytic rumen bacteria: their ecology and functions. Asian Austral J Anim 2009;22(1):131–8.

Lesmeister KE, Tozer PR, Heinrichs AJ. Development and analysis of a rumen tissue sampling procedure. J Dairy Sci 2004;87:1336–44.

Li RW, Wu S, Baldwin RL, Li W, Li C. Perturbation dynamics of the rumen microbiota in response to exogenous butyrate. PLoS One 2012;7(1):e29392.

Li D, Xiang Z, He JT, Zhang LL, Bai KW, Xu W, Wang T, Huang XX. Supplementation of tributyrin improves the growth and intestinal digestive and barrier functions in intrauterine growth-restricted piglets. Clin Nutr 2016;35(2):399–407.

Li Y, Wang H, Zhang Y, Li X, Jiang X, Ding H. Effects of dietary supplementation with glycerol monolaurate (GML) or the combination of GML and tributyrin on growth performance and rumen microbiome of weaned lambs. Animals 2022;12(10):1309.

Lin LM, Xie F, Sun DM, Liu JH, Wu WY, Mao SY. Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. Microbiome 2019;7(1):1–16.

Liu LX, Sun DM, Mao SY, Zhu WY, Liu JH. Infusion of sodium butyrate promotes rumen papillae growth and enhances expression of genes related to rumen epithelial VFA uptake and metabolism in neonatal twin lambs. J Anim Sci 2019;97(2):909–21.

Liu S, Ma YL, Zhou J, Wu JD, Li JH, Alugongo GM, Xiao JX, Wang JJ, Wang YJ, Wang W, Li SL, Cao ZJ. Tributyrin supplementation in pasteurized waste milk: effects on growth performance, health, and blood parameters of dairy calves. J Dairy Sci 2021;104:12496–507.

Liu S, Wu J, Wu Z, Alugongo GM, Khan MZ, Li JH, Xiao JX, He ZY, Ma YL, Li SL, Cao ZJ. Tributyrin administration improves intestinal development and health in pre-weaned dairy calves fed milk replacer. Animal Nutrition 2022;10:399–411.

McLeod KR, Baldwin RLVI. Effects of diet forage-to-concentrate ratio and metabolizable energy intake on visceral organ growth and in vitro oxidative capacity of gut tissues in sheep. J Anim Sci 2000;78:760–70.

Mekbungwan A, Yamauchi KE, Thongwittaya N. Intestinal morphology and enteral nutrient absorption of pigeon pea seed meal in piglets. Anim Sci J 2002;73:509–16.

Mentschel J, Leiser R, Mülling C, Pfarrer C, Claus R. Butyric acid stimulates rumen mucosa development in the calf mainly by a reduction of apoptosis. Arch Anim Nutr 2001;55:85–102.

Ministry of Agriculture and Rural Affairs (MARA) of the People's Republic of China. Operating procedures of livestock and poultry-Sheep and goat. Beijing, China: Agricultural Standard Publishing Research Center; 2019. p. 1–6.

Miyoshi M, Sakaki H, Usami M, Iizuka N, Shuno K, Aoyama M, Usami Y. Oral administration of tributyrin increases concentration of butyrate in the portal vein and prevents lipopolysaccharide-induced liver injury in rats. Clin Nutr 2011;30(2):252–8.

Murayama K, Fukui T, Kushibiki S, Sakamoto K, Inouchi K, Sugino T. Effects of medium-chain fatty acids and tributyrin supplementation in milk replacers on growth performance, blood metabolites, and hormone concentrations in Holstein dairy calves. J Dairy Sci 2023:106. https://doi.org/10.3168/jds.2022-22957.

Muscato TV, Tedeschi LO, Russell JB. The effect of ruminal fluid preparations on the growth and health of newborn, milk-fed dairy calves. J Dairy Sci 2002;85:648–56.

Niwińska B, Hanczakowska E, Arciszewski MB, Klebaniuk R. Review: exogenous butyrate: implications for the functional development of ruminal epithelium and calf performance. Animal 2017;11(9):1522–30.

NRC (National Research Council). Nutrition requirements of dairy cattle. Washington, D C: The National Academy Press; 2001. p. 1–381.

Ren QC, Xuan JJ, Wang LK, Hu ZZ, Yang HJ, Zhang W, Jiang LS. Effects of tributyrin supplementation on in vitro culture fermentation and methanogenesis and in vivo dietary nitrogen, calcium and phosphorus losses in Small Tail ewes. Anim Feed Sci Technol 2018a;243:64–71.

Ren QC, Xuan JJ, Hu ZZ, Wang LK, Zhan QW, Dai SF, Li SH, Yang HJ, Zhang W, Jiang LS. Effects of tributyrin supplementation on short-chain fatty acid concentration, fibrolytic enzyme activity, nutrient digestibility and methanogenesis in adult Small Tail ewes. J Agr Sci-. Camb Times 2018b;156(3):465–70.

Ren QC, Xuan JJ, Wang LK, Zhan QW, Yin DZ, Hu ZZ, Yang HJ, Zhang W, Jiang LS. Effects of tributyrin supplementation on ruminal microbial protein yield, fermentation characteristics and nutrients degradability in adult Small Tail ewes. Anim Sci J 2018c;89(9):1271–9.

Ren QC, Xuan JJ, Che CY, Yan XC, Hu ZZ. Desirable effects of dietary 4-O-methylglucuronoarabinoxylan from the Echinacea plant on growth performance, thigh meat quality and development of small intestine in female Partridge-Shank broilers. Anim Prod Sci 2020;60(2):288–95.

Song ZH, Xuan JJ, Ren QC. Effects of dietary tributyrin supplementation on in vitro fermentation characteristics of ruminal microbes. Journal of Anhui Science and Technology University 2020;34(4):6–12 (*in Chinese*).

Sun DM, Yin YY, Guo CZ, Liu LX, Mao SY, Zhu WY, Liu JH. Transcriptomic analysis reveals the molecular mechanisms of rumen wall morphological and functional development induced by different solid diet introduction in a lamb model. J Anim Sci Biotechnol 2021;12(1):1–15.

Swiatkiewicz M, Hanczakowska E. Effect of crude fiber concentrate supplementation on some characteristics of piglet intestine. Polish J Nat Sci Suppl 2006;3:377–82.

Tan P, Liu H, Zhao J, Gu X, Wei X, Zhang X, Ma N, Johnston LJ, Bai YY, Zhang WJ, Nie CX, Ma X. Amino acids metabolism by rumen microorganisms: nutrition and ecology strategies to reduce nitrogen emissions from the inside to the outside. Sci Total Environ 2021;800:149596.

Van Soest PJ, Robertson JB, Lewis BA. Carbohydrate methodology, metabolism, and nutritional implications in dairy cattle: methods for dietary fibre, neutral detergent fibre, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74:3583–97.

Wheeler TT, Haigh BJ, Broadhurst MK, Hood KA, Maqbool NJ. The BPI-like/PLUNC family proteins in cattle. Biochem Soc Trans 2011;39:1006–11.

Wilkens MR, Muscher-Banse AS. Review: regulation of gastrointestinal and renal transport of calcium and phosphorus in ruminants. Animal 2020;14:s29–43.

Yeoman CJ, White BA. Gastrointestinal tract microbiota and probiotics in production animals. Annu Rev Anim Biosci 2014;2(1):469–86.

Zhang B, Liu MQ, Yue ZK, Chen XY, Li CY, Liu L, Li FC. Combined omics analysis further unveils the specific role of butyrate in promoting growth in early-weaning animals. Int J Mol Sci 2023;24(2):1787.

Zhong HY, Yu WJ, Wang M, Lin B, Sun XZ, Zheng N, Wang JQ, Zhao SG. Sodium butyrate promotes gastrointestinal development of preweaning bull calves via inhibiting inflammation, balancing nutrient metabolism, and optimizing microbial community functions. Animal Nutrition 2023. NINU 733. https://doi.org/10.1016/j.aninu.2023.04.004.