Scientific Research Publishing

Food and Nutrition Sciences, 2024, 15, 1065-1084
https://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

Tributyrin in the Diet of Lambs and Its Impacts on Energy Metabolism and Oxidative Status in the Liver and Intestine, as Well as the Fatty Acid Profile in Meat

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How to cite this paper: dos Santos, A.I.S., Kessler, J.D., Klein, B., Brunetto, A.L.R., Deolindo, G.L., Silva, L.E.L., Wagner, R., da Silva, P.M., Baldissera, M.D. and da Silva, A.S. (2024) Tributyrin in the Diet of Lambs and Its Impacts on Energy Metabolism and Oxidative Status in the Liver and Intestine, as Well as the Fatty Acid Profile in Meat. *Food and Nutrition Sciences*, **15**, 1065-1084.
https://doi.org/10.4236/fns.2024.1511069

Received: September 16, 2024
Accepted: November 9, 2024
Published: November 12, 2024

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Abstract

This study aimed to determine whether adding tributyrin to the diet of lambs impacts health, energy metabolism, ruminal environment, and meat quality. Twelve lambs were used; the control group received a basal diet, while the tributyrin group received a diet with the additive mixed with concentrate (2 g/day/animal). The blood count was used for Leukocyte and lymphocyte counts, which were significantly higher in tributyrin-fed animals than in controls. The activity of the enzymes adenylate kinase and pyruvate kinase was higher in the liver and intestine of the tributyrin group than the controls; cytosolic creatine kinase activity was significantly higher in the intestine of lambs fed tributyrin. Glutathione S-transferase activity in the liver was significantly higher in animals in the tributyrin group. Superoxide dismutase activity was significantly higher in the intestine, with a lower protein carbonyl concentration in the tributyrin group. Bacterial activity through ruminal fluid collection was significantly lower when tributyrin was consumed, unlike the protozoan count, which was significantly higher in animals in the tributyrin group than in the controls. Tributyrin intake caused lower levels of short-chain fatty acids without changing the proportion of volatile fatty acids. The water retention capacity measured using an external compression method was significantly higher in the meat of the tributyrin group. The treatment affected some fatty acids in

the meat, these acids were separated by chromatography where a lower amount of saturated fatty acids and a higher amount of monounsaturated fatty acids in the group that consumed tributyrin. These findings suggest that tributyrin in lamb diet alters blood and rumen environment biomarkers and improves the fatty acid profile of the meat.

Keywords

Additive, Animal Production, Tributyrin, Fatty Acids, Animal Health

1. Introduction

In its pure form, butyrate has an unpleasant odor, with volatile and unstable behavior in the environment [1] to avoid these problems, butyric acid molecules are esterified to mono-, di-, and tributyrates. Compared to butyric acid, tributyrin (TB) is more stable and has less odor, thus allowing a slower release to reach the lower gut and eliminating the possibility of animal consumption rejection. TB encompasses a complex interplay between diet, gut microbiota, energy metabolism, and immunity [2]-[4]. In recent years, it has attracted much attention and is widely used as a food additive in diets to improve the health and performance of farm animals, as it can be metabolized into three free butyric acid molecules in the gastrointestinal tract. In adult sheep, the addition of TB improved the ruminal environment and metabolites, thus resulting in greater activity of fibrolytic bacteria and utilization of dietary nutrients [5] Butyrate recruits innate immunity molecules [6] [7]. In pre-weaned dairy calves, added to milk replacer promoted intestinal development by increasing the intestinal barrier and attenuating the inflammatory response [8] [9].

In practice, TB is used in the production of swine [10], poultry [9] [11], and aquatic organisms [12]-[14] as it is a potent growth promoter with effective antibiotic functions. However, the effects of TB on sheep diets are relatively unknown. In recently weaned lambs, a basal diet with additives of mono-, di-, and tri-glycerols allowed researchers to attribute a positive role to TB in the degradation of ruminal fiber, which combined with other esters of glycerol, increased average daily weight gain, even without an effect on the rumen microbiome. On the other hand [15], evaluated various doses of protected or unprotected TB in the diet of sheep and did not observe a contribution to growth, final weight, average daily gain (ADG), gain:feed ratio, hot carcass yield, or carcass yield. We hypothesize that TB will alter the flora and profile of AVGs in the rumen environment and reach the intestine, where enterocytes will use it, favoring intestinal and animal health. Therefore, this study aimed to determine whether adding TB to the diet of lambs impacts animal health, energy metabolism, rumen environment, and meat quality.

2. Materials and Methods

2.1. Tributyrin

The TB additive used in this study was a commercial product based on butyric acid glyceride molecules (Villonat®, Feedis, Salto, São Paulo, Brazil).

2.2. Animals, Data, and Sample Collection

The experiment was conducted on an Experimental Farm of the Center for Higher Education of the West of the State University of Santa Catarina, in Guatambu (Southern Brazil). FECEO-UDESC at latitude 27˚8'5" South, longitude 52˚47'15" W between March and June 2023.

Twelve castrated male Lacaune lambs (2.5 ± 0.5 months old and 21 ± 2 kg) were housed in 3-m^2 wooden pens with individual drinking and feeders for 79 days. The distribution of the stalls in the respective treatments in the installation was sequential to avoid environmental interference. All animals were treated preventively with Ripercol anthelmintic based on 5% levamisole hydrochloride (Ripercol®).

The basal diet was formulated with nutritional requirements for growing lambs, according to NRC 2007. Feed was provided twice daily (08:30 am and 3:30 pm) in a 75:25 concentrate:roughage ratio. The amount of feed provided to the animals was adjusted whenever the animals were weighed, with the total mixed ration (TMR) corresponding to 4% of live weight (Table 1).

From the record of the offer and refusal of daily feed, dry matter intake (DMI), feed conversion (weight animal weight in several studies: gain/DMI), and feed efficiency per pen were calculated; for these zootechnical variables, the pen was used as an experimental unit. The additive was adjusted accordingly, ensuring that the animals always ingested 2 g/day/animal defined by the source of tributyrin or butyric acid and the average First, the concentrate was supplied after consumption, and the bulky portion (corn silage) was supplied to guarantee maximum additive consumption.

To evaluate performance, in addition to weighing, weight gain and average daily weight gain were calculated five times on a digital scale (days 1, 15, 35, 45, and 70). The leftovers were weighed before the day's first batch was delivered. Based on this data, feed conversion, and feed efficiency were calculated.

2.3. Hematology and Serum Biochemistry

Blood was collected on days 1, 15, 35, 45, and 70 days of the experimental period by puncture of the jugular vein of fasting animals using two types of vacuolated tube: without anticoagulant and with EDTA as anticoagulant; samples were transported refrigerated for serum biochemistry and complete blood counts, respectively.

Blood collected without anticoagulant was centrifuged at 7,000 rpm for 10 min to separate the serum and stored at −20˚C until biochemical assays were performed. Serum activity of aspartate aminotransferase (AST), gamma-glutamyl transferase

Table 1. Ingredients and chemical composition of the experimental diet concentrate.

Ingredients (g/kg)	**Composition**		
Corn meal	506.00		
Soybean meal	246.00		
Wheat bran	180.00		
Mineral core[1]	47.00		
Calcitic limestone	20.00		
Chemical composition[2]		**Control**	**Tributyrin**
Items	**Corn silage**	**Concentrate**	**Concentrate**
DM	34.00	89.64	89.87
MM	4.60	12.01	12.37
CP	8.78	22.00	22.88
NDF	25.60	14.77	14.01
ADF	13.70	6.66	6.22
EE	2.10	3.26	3.24

[1]Nucleus composition: Calcium (min./max.) 175/245 g; Phosphorus (min.) 40 g; Sulfur (min.) 25 g; Magnesium (min.) 12 g; Sodium (max) 100 g. Mineral Matter (max) 980 g; Cobalt (min.) 40 mg; Iron The animals were allocated to two experimental groups: 8 received concentrated feed containing 2 g/day/animal of the TB additive, while another 8 received concentrated feed without the additive. The dose of TB added to the concentrate during its production was based on the concentrate/day consumed. Corn silage and concentrate samples were collected to determine the chemical composition of dry matter (DM), crude protein (CP), ether extract (EE), and ash according to AOAC (2000) (Table 1). (min.) 1,800 mg; Iodine (min.) 80 mg; Manganese (min.) 1,400 mg; Selenium (min.) 12 mg; Zinc (min.) 4,000 mg; Vit. A (min.) 300,000 IU; Vit. D3 (min.) 60,000 IU; Vit. E (min.) 600 IU; Fluoride (max) 600 mg; Monensin sodium (600 mg); [2]DM (dry matter), MM (mineral matter), CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), EE (ether extract). Note: Basal concentrate, used in all groups. The chemical composition of the concentrate from both groups was the same; TB did not change the chemical composition of the concentrate consumed by the lambs.

(GGT), and levels of total protein, albumin, glucose, triglycerides, and cholesterol, calcium, phosphorus were measured on a Zybio EXC-200 model analyzer using commercial kits (Analisa®, Belo Horizonte, Brazil) according to the manufacturer's recommendations. Globulin levels were calculated as total protein – albumin.

Hematologic analyses were performed with whole blood stored in tubes containing EDTA within two hours of collection. An automatic hematologic analyzer measured the erythrocyte count, total leukocyte count, leukocyte differential, hematocrit percentage, and hemoglobin concentration (3-part EQUIP VET 3000®).

2.4. Rumen Parameters

Ruminal fluid (50 ml) was collected on days 1, 35, and 70 (two hours after feeding in the morning) through a silicone ororuminal cannula connected to a vacuum pump. Half of the fluid (25 ml) was filtered for pH assessments using a portable digital potentiometer (TESTO 205®) and measuring the functional activity of the

ruminal microbiota using the methylene blue reduction test—MBRT (0.05% solution), as described by [16]. The methylene blue reduction time was measured accordingly. From this filtered portion, 2 mL was used to determine short-chain fatty acids (SCFA), and the proportion of acetic, propionic, butyric, valeric, and isovaleric acids using a gas chromatograph with a flame ionization detector (GC-FID; Varian Star 3600, Palo Alto, USA) and automatic sampler (Varian 8200CX, Palo Alto, USA), according with adjusted detail in **Supplementary Material 1**. The results were expressed as mmol·L^{-1} of each SCFA in ruminal fluid.

An unfiltered aliquot was used in the protozoan counting process using a Sedgewick Rafte chamber, where in one drop of dilution (10^{-1}), protozoan microstructures are counted with a 40x objective according to literature [4].

2.5. Post-Mortem Analysis

2.5.1. Slaughter and Sample Collection

Once the confinement phase (79 days) was completed, the twelve lambs were fasted for 12 hours, and transported to the slaughterhouse following local regulations. After bleeding, skinning, and eviscerating the animals, the liver, jejunum, and longissimus thoracis (LT) muscle were collected for the analyses described below.

2.5.2. Energy Metabolism and Oxidative Status in the Liver and Intestine

The intestine was washed in SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4) and homogenized in the same buffer (1:10 w/v) using a Potter tissue homogenizer. The homogenate was centrifuged at 800 × *g* for ten minutes at 4˚C. An aliquot of the supernatant was used to evaluate adenine kinase (AK) activity; the pellet was discarded, and the supernatant was centrifuged again at 10,000 × *g* for 15 min at 4˚C. The cytosol supernatant was collected to determine cytosolic pyruvate kinase (PK) and creatine kinase (CK) activity. The pellet containing mitochondria was washed twice with the same buffer and resuspended in 100 mM Trizma and 15 mM $MgSO_4$ buffer (pH 7.5) to measure mitochondrial CK activity.

CK intestinal activity was conducted based on Hughes's colorimetric method (1962), which estimates creatine levels at a wavelength of 540 nm, as reported in detail by researchers [17]. The results were expressed as nmol of creatine formed/min/mg of protein. Intestine and liver AK activity was measured with an enzymatic assay coupled with hexokinase (HK) and glucose 6-phosphate dehydrogenase [17] [18]. The activity was expressed as pmol of ATP formed/min/mg of protein. Gut and liver PK activity were published in detail by researchers [17]. Activity was expressed as μmol of pyruvate formed/min/mg of protein.

Detection of serum lipid peroxidation was performed indirectly and expressed as serum thiobarbituric acid reactive substance (TBARS), according to [18] Serum glutathione S-transferase (GST) activity was measured using the technique described [19]. Results were expressed as μmol CDNB/min/mg protein.

Superoxide dismutase (SOD) activity was determined according to the

principle of pyrogallol autooxidation, which inhibits the presence of SOD. The variation in optical density was determined kinetically for two minutes at 420 nm, in 10-s intervals, according to the methodology described by researchers [20]. SOD activity was expressed as U SOD/mg protein.

2.5.3. Carcass Characteristics and Meat Quality

Measuring pH with a portable pH meter equipped with a penetrating electrode (Testo® model 205) in carcasses' loins (LT) immediately after evisceration, then individually weighing the carcass components on a digital scale. These were cooled at 4˚C for 24 h to obtain cold carcass weight (CCW); in the cold carcass, pH was also measured. Hot and cold carcass yields were calculated by expressing hot carcass weight (HCW) and CCW as a percentage of empty body weight (EBW), being (EBWZ = slaughter BW − [HCW OR CCW * 100]). Subsequently, the carcasses were ribbed in the twelfth and thirteenth to obtain the LT muscle, and stored at −18˚C until analysis of the chemical composition and fatty acid profile of the meat.

2.5.4. Color and Water Retention Capacity

The LT muscle was used to evaluate meat quality characteristics immediately upon arrival in the laboratory. Color analysis was performed using a portable spectrophotometer model CM-25d (Konika Minolta®) placed on the surface of the muscle to record brightness (L*), redness (a*), and yellowness (b*). All meat quality measurements were made in triplicate for each lamb.

For water retention capacity (WRC), a circular piece of meat (0.05 kg) from LT muscle was weighed on a precision scale and subjected to a rapid absorptive method of external compression, where the meat was pressed between two paper filters and loaded with a 2-kg block for five minutes, as detailed by researchers [21]. The WRC value was expressed as a percentage: WRC (%) = (Final weight − initial weight) × 100.

2.5.5. Chemical Composition and Fatty Acid Profile

The LT muscle samples were thawed to detail the chemical composition of the meat obtained using a near-infrared reflectance spectrometer, model Spectra Star 2600 XT series of Near-Infrared Analyzers (Unity Scientific®).

Fatty acid profiling, intramuscular fat extraction, methylation, and fatty acid profile analysis on LT samples were performed according to literature [22] as follows: at 15 mL polypropylene tube was added 1.5 g of ground muscle, 0.5 mL of distilled water, 5 mL of methanol, and 2.5 mL of chloroform to undergo mechanical agitation for 1 hour and 30 minutes, thus forming a homogeneous solution. Next, 2.5 mL of chloroform and 2.5 mL of 1.5% sodium sulfate (Na_2SO_4) solution were added to promote a biphasic solution and undergo mechanical agitation for 30 minutes, then centrifuged for 10 minutes at 2000 rpm. The lipids in the lower phase of the solution were subjected to fat quantification by gravimetric method and fatty acid analysis. For fat, 2.5 mL of the obtained solution was placed in a crucible and subjected to an oven for 45 minutes at 105˚C; the crucible was then

weighed to quantify the fat after evaporation of the chloroform.

The fatty acids (FA) methylation was from a previously obtained solution using quantitative method [21]. In a glass tube with a lid, 0.8 mL of solution in 1 mL of 0.4 M KOH in methanol was added, kept in a water bath at 100˚C for 10 minutes, and cooled to room temperature. Then, 3 mL of 1 M H_2SO_4 in methanol was added and kept in a water bath at 100˚C for ten minutes. After cooling, 2 mL of hexane was added, vortexed for two minutes, and centrifuged at 2000 rpm for 10 min. Finally, 1 µL of hexane with fatty acid methyl esters (FAME) was subjected to chromatographic analysis.

A gas chromatograph model TRACE 1310 was used to characterize FAME, equipped with a flame ionization detector (Thermo Scientific) with hydrogen used as a carrier gas at a constant flow of 1.5 mL/min. One microliter of sample was injected into a split-splitless injector, operated in split mode with a 1:10 ratio at 250˚C.

FAME separation used an RT 2560 chromatography column (100 m × 0.25 mm × 0.20 µm film thickness, Restek, USA). The initial oven temperature was programmed at 100˚C for 5 min and increased to 180˚C at an 8˚C/min rate. Then, it increases to 210˚C at a rate of 4˚C /min, and finally up to 240˚C, increasing by 20˚C/min and maintaining for 20 min at isothermal conditions. The detector temperature was kept constant at 250˚C.

Fatty acid values were expressed as a percentage (%) determined by comparing the total amount of FA to the authentic standard (MIX-37, Sigma Aldrich, St. Lois, MO). The percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MIFA), and polyunsaturated fatty acids (PUFA) were calculated.

2.6. Statistical Analysis

The design was in a completely randomized model where each pen was considered an experimental unit. Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene tests, respectively. The complete blood count results, quantification of protozoa in ruminal fluid, and the oxidative status of tissues required transformation to achieve normality and homogeneity and they were then retransformed to the original units for description. All data were analyzed using the SAS "MIXED procedure" (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects test. Growth performance data (exception for PC) were tested for fixed treatment effects using animal (treatment) as a random effect. Body weights, complete blood counts, and serum biochemical data were analyzed as repeated replicates. All data obtained on day 1 for each variable were included as covariates in each analysis. The first-order autoregressive covariance structure was selected according to Akaike's least information criterion. Means were separated using the PDIFF method, which selected a t-test, and all results were expressed as means with standard errors. Between treatments, differences were defined when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

3. Results

3.1. Performance

There was no effect of treatment and treatment x day interaction for body weight of lambs (Table 2). TB intake did not enhance weight gain, alter DMI, or improve feed conversion and efficiency (Table 2).

Table 2. Zootechnical performance of lambs fed with tributyrin.

Items	Treatments		SEM	*P*-value
	Control	Tributyrin		
Weight gain (kg)				
Adaptation period (10 days)	20.2	19.8	0.36	0.86
D1	23.9	23.8	0.33	0.94
D15	28.6	27.7	0.35	0.92
D35	33.9	33.2	0.35	0.95
D53	37.4	37.9	0.46	0.97
D70	44.1	44.2	0.41	0.97
Days 1 to 70 (trial period)	20.2	20.4	0.23	0.96
Average daily gain (ADG), kg/day	0.288	0.291	0.05	0.94
Dry matter intake (DMI). kg/day	1.37	1.38	0.11	0.96
Feed conversion (DMI/ADG). kg/kg	4.75	4.77	0.18	0.94
Feed efficiency (ADG/DMI). kg/kg	0.210	0.210	0.01	0.98

3.2. Hematology and Serum Biochemistry

Table 3 displays the results of hematologic and biochemical variables. Treatment had a significant effect on leukocyte count (P = 0.045). There was a significant effect of treatment (P = 0.022) and a tendency for treatment x day interaction (P = 0.080) for lymphocyte count, being significantly higher in TB animals compared to control. No significant effect of treatment and treatment x day interaction was observed for monocyte, granulocyte, erythrocyte, platelet counts, or hemoglobin concentration and hematocrit percentage.

There was a trend toward a treatment effect for serum glucose concentration, which was significantly higher in the blood of animals that consumed TB. No significant effect was observed for treatment and treatment x day interaction for AST, GGT, total protein, cholesterol, albumin, globulin, calcium, or phosphorus.

3.3. Ruminal Indicators

The results of biomarkers in the rumen environment are displayed in Table 4.

Table 3. Serum biochemistry and blood count of lambs fed tributyrin mixed with concentrate.

Variable[1]	Treatments				
			SEM	*P*-value	
Biochemistry	**Control**	**Tributyrin**		**Treat.**	**Treat. × day**
AST (U/L)	95.59	94.02	2.659	0.930	0.912
GGT (U /L)	64.97	65.13	2.347	0.898	0.941
Total prot. (g/dL)	6.48	6.55	0.088	0.923	0.881
Cholesterol (mg/dL)	79.24	78.72	1.505	0.906	0.784
Glucose (mg/dL)	93.97	99.4	1.065	**0.091**	0.141
Albumin (g/dL)	3.36	3.35	0.056	0.953	0.932
Globulin (g/dL)	3.11	3.20	0.104	0.947	0.956
Calcium (mg/dL)	11.31	11.42	0.228	0.970	0.970
Phosphorus (mg/dL)	10.60	10.58	0.303	0.962	0.947
Hemogram (μ/L)					
Leukocytes	7.470[b]	10.33[a]	0.951	**0.045***	0.105
Lymphocytes	5.380[b]	7.727[a]	0.316	**0.022***	**0.080***
Monocytes	0.621	0.901	0.242	0.180	0.207
Granulocytes	1.469	1.706	0.155	0.274	0.398
Erythrocytes	10.10	10.42	0.166	0.965	0.981
Hemoglobin (g/dL)	12.44	12.61	0.240	0.950	0.963
Hematocrit (%)	27.83	27.54	0.240	0.951	0.968
Platelets	282.8	239.9	20.80	0.694	0.725

[1]AST (aspartate aminotransferase), GGT (gamma-glutamyl-transferase). *Within the lines, differ (P ≤ 0.05) or tend to differ (P ≤ 0.10).

Table 4. Ruminal parameters of lambs fed tributyrin mixed with concentrate.

Variáveis[1]	Treatments				
	Control	Tributyrin	EPM	*P*-value	
				Treatment	Treat × Day
pH ruminal fluid	6.243	6.403	0.03	0.920	0.950
MBRT[1]	92.05[b]	118.5[a]	6.21	**0.031**	0.128
Protozoans (nº/L)				0.253	**0.050***
D1	131.7	133.5	9.66		
D35	100.2	136.4	9.57		
D70	110.7[b]	131.4[a]	4.26		
SCFA (mnol/L)				**0.02***	**0.01***
D1	83.4	83.8	0.36		
D35	74.1[a]	62.8[b]	0.34		
D70	90.5[a]	85.1[b]	0.33		

Continued

Acetic acid (%)	59.2	59.4	0.81	0.95	0.97
Propionic acid (%)	28.4	27.7	0.34	0.96	0.92
Butyric acid (%)	8.49	8.69	0.12	0.93	0.95
Isovaleric acid (%)	1.59	1.69	0.03	0.82	0.76
Valeric acid (%)	2.23	2.42	0.06	0.88	0.71
Acetic/Propionic	2.24	2.23	0.05	0.97	0.95

[1]MBRT (methylene blue reduction test). *Within the lines, differ ($P \le 0.05$) or tend to differ ($P \le 0.10$).

There was no significant effect of treatment for pH; however, there was a significant effect of treatment for MBRT, with a longer time for consumption of methylene blue by bacteria, indirectly indicating lower microbial activity in the rumen environment when lambs consumed TB but without treatment x day interaction for MBRT. There was a significant treatment x day interaction on day 70 (P = 0.50) for protozoa counts in rumen fluid, with the number being significantly higher in lambs that consumed TB.

There was a significant effect of treatment and treatment x day interaction for concentrations of short FA, being significantly lower in lambs that consumed TB on days 35 and 70 (Table 4). We did not find a significant effect of treatment on the proportion of volatile FA called acetic, propionic, butyric, valeric, or isovaleric acids (Table 4; P > 0.05).

3.4. Energetic Metabolism and Oxidative Status

Table 5 displays the activity of energy metabolism enzymes and oxidative status in the liver and intestine. Treatment significantly affected AK activity in the liver (P = 0.05) and intestine (P = 0.01) PK activity. AK and PK significantly reduced their activities in the livers and intestines of lambs that consumed TB compared to the control group. In the intestine, there was a significant effect of treatment on cytosolic CK activity (P = 0.02), significantly higher in the jejunum of lambs in the TB group; Mitochondrial CK activity tended to be lower in the jejunum of animals in the TB group (P = 0.08).

There was no effect on protein carbonyl in the liver; however, there was in the intestine. That is, the concentration of this biomarker of protein oxidation was lower in the jejunum of lambs that consumed TB (Table 5). Treatment affected GST activity in the liver (P = 0.01), with the highest activity in lambs in the TB group; however, there was no change in this enzyme in the jejunum of the animals. There was no effect of treatment on SOD activity in the liver; however, in the intestine/jejunum, the activity of this enzyme was higher (P = 0.01) in lambs that consumed TB than control animals. It had no treatment effect for TBARS levels in the liver or intestine.

Table 5. Energy metabolism and oxidative status of the liver and intestine of lambs fed with tributyrin mixed with concentrate.

Variable[1]	Treatments			
	Control	Tributyrin	SEM	*P-value*
Liver				
AK	9.98[a]	7.95[b]	0.29	0.05*
PK	0.56[a]	0.47[b]	0.01	0.01*
TBARS	1.85	1.82	0.24	0.92
Carbonyl protein	3.68	3.47	0.63	0.73
GST (U/L)	152[b]	274[a]	5.89	0.01*
SOD (U/mg)	12.8	14.6	0.95	0.56
Jejunum				
AK	9.29[a]	6.35[b]	0.42	0.01*
PK	0.86[a]	0.59[b]	0.06	0.05*
(CK-C)[2] (nmol/mg)	1.85[b]	2.50[a]	0.07	0.02*
(CK-M)[2] (nmol/mg)	3.92[a]	3.38[b]	0.07	0.08*
TBARS	2.64	2.25	0.13	0.43
Carbonyl protein	4.58[a]	3.27[b]	0.15	0.02*
GST (U/L)	46.3	51.2	2.84	0.55
SOD[1] (U/mg)	8.56[b]	11.7[a]	0.89	0.01*

[1]cytosolic creatine kinase (CK-C), mitochondrial creatine kinase (CK-M), thiobarbituric reactive substances (TBARS), glutathione S-transferase (GST), superoxide dismutase (SOD), adenylate kinase (AK), pyruvate kinase (PK). [2]nmol of creatine formed per min per mg of protein (CK-C e CK-M), nmol of pyruvate formed per min per mg of protein (PK). *Within the lines, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$).

3.5. Carcass and Meat

Carcass results are presented in Table 6. There were no significant differences between treatments in carcass yield or pH in hot and cold carcasses. There were no significant differences between groups for the color characteristics (brightness, red color, and yellow color) or chemical composition (protein, fat, and organic matter) of the meat (Table 7). However, WRC was significantly higher in meat from TB-fed animals (Table 7; P = 0.050).

Results of the fatty acid profile in meat are displayed in Table 8. There was a significant effect of the treatment for the sum of SFA (P = 0.046) and monounsaturated fatty acids (MUFA) (P = 0.050), with lower SFA and higher MUFA in animals in the TB group. In the meat of animals in the TB group, there was a higher proportion of oleic acid (P = 0.041) and omega-3 eicosapentaenoic acid (P = 0.042), in addition to a tendency toward a higher percentage of linoleic acid (P = 0.074). The animals that consumed TB had a lower proportion of palmitic acid (P = 0.001) and a tendency toward a lower percentage of palmitoleic acid (P = 0.091). The other variables determined in chromatography did not differ between groups and are presented in Table 8.

Table 6. Carcass parameters of lambs fed tributyrin mixed with concentrate.

Variables[1]	Treatment			
	Control	Tributyrin	EPM	*P*-value*
HCW (%. kg)	47.4	47.5	0.051	0.917
CCW (%. kg)	43.5	44.3	0.034	0.183
pH HCW	6.88	6.86	0.090	0.951
pH CCW	6.21	6.47	0.314	0.804

[1]HCW (hot carcass weight), CCW (cold carcass weight). Hot carcass weight (HCW), cold carcass weight (CCW). *Within the lines, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$).

Table 7. Meat quality of lambs fed with tributyrin mixed with concentrate.

Variables[1]	Treatment			
	Control	Tributyrin	EPM	*P*-value
WRC (%)	84.8^{b}	87.7^{a}	1.562	**0.050**
Cor				
Luminosity (L)	35.9	34.3	0.548	0.625
Redness (a)	10.8	9.35	0.420	0.502
Yellowness (b)	9.72	8.42	0.340	0.752
Chemical composition (%)				
Moisture	71.9	72.4	0.952	0.909
Total protein	22.2	21.6	0.812	0.892
Total fat	2.61	2.62	0.625	0.974

[1]WRC (water retention capacity). $^{a-b}$Within the lines, unusual subscripts differ ($P \leq 0.05$) or tend to differ.

Table 8. Fatty acid profile of lamb meat fed with tributyrin mixed with concentrate.

Variables	Treatment			
FA (%)	Control	Tributyrin	SEM	P-value
C10:0 (Capric)	0.068	0.065	0.003	0.951
C12:0 (Lauric)	0.080	0.083	0.003	0.957
C14:0 (Myristic)	1.937	1.766	0.067	0.428
C14:1 (Myristoleic)	0.088	0.093	0.018	0.921
C15:0 (Pentadecanoic)	0.305	0.317	0.033	0.938
C16:0 (Palmitic)	26.115^{a}	23.902^{b}	0.306	0.001*
C16:1 (Palmitoleic)	1.948^{a}	1.605^{b}	0.061	0.091*
C17:0 (Heptadecanoic)	1.175	1.076	0.045	0.820
C18:0 (Stearic)	15.325	15.718	0.146	0.967
C18:1n9t (Elaidic)	1.381	1.393	0.091	0.983
C18:1n9c (Oleic)	43.18^{b}	45.02^{a}	0.350	0.041*

Continued

C18:2n6c (Linoleic)	4.774[b]	5.201[a]	0.177	0.074*
C20:0 (Arachidic)	0.132	0.115	0.003	0.873
C18:3n6 (?-Linolenic)	0.074	0.069	0.003	0.882
C20:1n9 (cis-11-Eicosenoic)	0.100	0.113	0.002	0.904
C18:3n3 (a-Linolenic)	0.176	0.198	0.008	0.913
C21:0 (Henicsanoic)	0.300	0.279	0.012	0.856
C20:2 (cis-11.14-Eicosadienoic)	0.043	0.048	0.002	0.899
C22:0 (Behenic)	0.026	0.031	0.002	0.621
C20:3n6 (cis-8.11.14-Eicosatrienoic)	0.155	0.166	0.009	0.795
C20:4n6 (Arachidonic)	2.459	2.566	0.122	0.823
C24:0 (Lignoceric)	0.027	0.030	0.002	0.941
C20:5n3 (cis-5.8.11.14.17-Eicosapentaenoic)	0.059[b]	0.072[a]	0.004	0.042*
C24:1n9 (Nervonic)	0.024	0.024	0.002	0.981
C22:6n3 (cis-4.7.10.13.16.1 Docosahexanoic)	0.042	0.042	0.003	0.978
Sum (%)[1]				
SFA	45.49[a]	43.38[b]	0.333	0.046*
MUFA	46.72[b]	48.25[a]	0.335	0.050*
PUFA	7.782	8.363	0.332	0.510

[1]Saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA). [a-b]Within the lines, unusual subscripts differ (P ≤ 0.05) or tend to differ.

4. Discussion

The addition of TB to the lamb diet had more marked effects on immune system cells, oxidative status, and fatty acid profile in the meat. Therefore, we need to understand the response mechanisms that were accentuated in these variables. SCFAs are believed to have a close relationship between diet, energy metabolism, ruminal and intestinal microbiota, and host immunity. SCFAs are used as energy and signaling for rumen development and immunomodulatory functions [3] [23]. However, its use in animal feed for ruminants is generally in protected form, which increases the costs of the additive, mainly due to the pungent odor that limits consumption in the free form for ruminants. In the present study, when butyric acid was used as TB, this did not limit the consumption of concentrate by the lambs. In another study, adult sheep had reduced food consumption when the additive TB was present [5] the trophic effect of exogenous TB on the gastrointestinal system is age-dependent in ruminants because butyrate stimulates IGF-1 and IGF-2 in young animals for rumen development, resulting in a greater absorptive surface in the rumen and better growth performance [8] [24]; this effect is not necessary for adult animals. The lambs in the present study were three months old when we started the study, although they had already adapted to the palatability

of the concentrate. According to researchers, the various butyric molecules are more effective in this early stage of the ruminants' life having effects on nutrient absorption and promoting animal development in the first months of life [25]; however, this effect does not persist or is no longer different from control animals over time [23] [26] [27]. Growth performance was analyzed in this study as a complementary variable, since our sample size was planned to study mechanisms and pathways of action of BT in lambs without differences between groups, although offering sodium butyrin in the diet of calves in the pre-weaning phase [8] and found an increase in growth performance while found positive modulations in the diversity of the intestinal microbiota [28]. On the other hand, researchers [1] used 0.03% of tributyrin or sodium butyrin per kg of DM consumed and found lower final weight and ADG/DMI of calves than the control. The same authors reported an increase in plasma glucose concentration in both phases of the study, suggesting an improvement in energy metabolism, corroborating our trend for higher glycemia levels.

Researchers who fed lambs with glycerol monolaurate, a medium-chain glyceride, combined with TB (3 g/kg DM) recorded changes in the rumen microbiome, highlighting a greater abundance of the phyla Bacteroidetes and Verrucomicrobia, the first group of bacteria being more generalized in the process. This phenomenon differed from the second group, which had an enzymatic system for breaking down more complex polysaccharides/fibers. Furthermore, these researchers found that this additive could increase the apparent digestibility of NDF [29] and observed an increase in the relative abundance of the phyla Firmicutes, Bacteroidetes, and Fibrobacteres in lambs that consumed TB. In the present study, we did not evaluate microbiota or digestibility but rather rumen bacterial activity, which was lower in the rumen fluid of lambs that consumed TB. Our finding might indicate the antimicrobial effects of the glyceride, a biological property that is well known. This lower bacterial activity may be directly related to the lower levels of volatile FA in the rumen of these lambs. Nevertheless, this is a hypothesis that needs to be tested.

In adult sheep and *in vitro*, researchers observed more significant activity of fibrolytic enzymes (kilase and carboxymethyl cellulase), a higher concentration of total volatile FA, and lower rumen pH in the presence of TB [5]. A class of molecules that links the microbiota and the immune system are volatile FA, and they even play an active role in the innate reactions of cattle [6] [30]. Butyrate requires the mobilization of Ca^{2+} with a particular concentration in plasma to produce cytokines [6]. However, in the present study, there were no changes in serum Ca^{2+} levels; at the evaluated moments, this relationship was not observed. However, there were changes in immune response biomarkers.

According to the literature, the stimulus that TB causes in the animal can alter the immune function of lambs, especially free butyrate fatty acids that are received by G/GPCR proteins (FFA2 AND FFA3) throughout the gastrointestinal tissue, acting on the expression of immunocompetent cells, such as neutrophils, and on

the mobilization of Ca^{2+} in organs such as the liver, small intestine (duodenum), and rumen [1]. These differential effects on immune cell functions at various times can be explained by two independent mechanisms [5]: a) activation of free fatty acid receptors, FFAR2 and FFAR3 [1] and b) inhibition of histone deacetylase (HDAC) [29].

SCFA are agonists mainly of FFA2 and receipt free fatty acids in an order according to the chain length (C4 > C3 > C2), as the length of the carbon chain is fundamental in the interaction of fatty acids and triglycerides with cells of the system immunological [29]. On the other hand, the mechanism in the modulation of butyrate immune responses is related to HDAC inhibition that occurs in macrophages and deserves to be investigated in future studies for TB.

In both tissues (liver and intestine), we observed effects on the oxidative status associated with changes in the activity of ATP metabolism enzymes, *i.e.*, AK, PK, and CK. The increase in cytosolic CK activity in the jejunum demonstrates that TB treatment stimulates ATP production and improves energy homeostasis since it is considered a central controller of cellular energy homeostasis activity through the reversible interconversion of creatine into phosphocreatine [31]. It is essential to report that CK accumulates a large pool of rapidly diffusing phosphocreatine for temporal and spatial buffering of ATP levels. Thus, CK is particularly critical in tissues with large and fluctuating energy demands, such as muscles, jejunum, and brain [17].

AK is involved in energy metabolism and homeostasis of cellular adenine nucleotide ratios in different intracellular compartments through catalyzing the reversible transfer of a γ-phosphate group from ATP to AMP, releasing two molecules of ADP [32] In the present study, the use of tributyrin reduced AK activity, indicating that treatment impairs liver energetic homeostasis, a condition that can be corroborated with reduced PK activity in the same tissue. It is essential to highlight that AK and PK activities were also reduced in jejune, but cytosolic CK activity increased, which can be considered a compensatory mechanism that aims at improving oxidative energy metabolism [18]. It is necessary to highlight a mutual compensatory relationship between CK, AK, and PK to safeguard the cellular energy economy, which contributes to efficient intracellular energetic communication to maintain the balance between cellular ATP use and synthesis [18]. Therefore, the use of TB improves energy metabolism in the jejunum by increasing cytosolic CK, while the reduction in AK and PK activity may be compensatory mechanisms for maintaining homeostasis.

Analyzing the intestinal and hepatic oxidative status, ingesting TB has an antioxidant effect. Other studies mentioned an activation of the activity of antioxidant enzymes combined with a reduction in oxidation reactions; however, the mechanisms involved are not clear; nevertheless, we believe that this phenomenon is related to improved animal health in general, primarily due to the anti-inflammatory effect provided by butyric acid glycerides. Researchers fed sodium butyrate via milk to calves and found that serum glutathione peroxidase activity increased linearly with increasing sodium butyrate levels, while serum maleic dialdehyde

concentration decreased linearly [8]. The researchers also evaluated the activity of CAT and SOD, but with no effect of treatment on both [8], unlike our study, where the activity of intestinal SOD was higher associated with lower protein oxidation in this organ. Furthermore, the present study observed more significant GST activity in the liver, a hepatic antioxidant enzyme critical for the organ's detoxification process.

In carcasses, our findings aligned with those of other authors [15], who evaluated different doses of protected or unprotected TB fed to lambs, *i.e.*, without effects on hot carcass yield and final carcass yield. However, in the present study, carcasses from animals fed with TB suffered fewer cooling losses; this is an excellent result and may be directly related to the antioxidant response of TB, which leads to cell protection. However, this finding can also represent a negative effect if we consider that losing less water can make it difficult to adequately reduce pH, which is necessary for muscle transformation and maturation.

Like other variables, the color, chemical composition, and fatty acid profile of lamb can be influenced by nutrition and production systems [33] [34]. Therefore, many nutritionists have used this knowledge to produce healthier foods, as discussed below. In the present study, eicosapentaenoic (omega n3) and linoleic (omega C18:2n-6) fatty acids were in more significant proportion in the meat of lambs that consumed TB; when added to other unsaturated substances, meat showed a higher proportion of MUFAs, while the levels of SFA were lower, making the meat more sensitive to oxidation but healthier for humans. Several risk factors for cardiovascular diseases are beneficially modified by increased intake of MUFAs, including blood pressure, platelet reactivity, thrombosis, plasma concentrations of triacylglycerols, vascular function, cardiac arrhythmias, blood flow variability, heart rate, and inflammation [35]. Because the VFA profile results were discreet in rumen fluid, with a lack of treatment effect on serum biomarkers of lipid metabolism, we could not determine the mechanism involved in the changes in the fatty acid profile in the meat of lambs that consumed TB.

5. Conclusion

Intake of 2 grams of TB/day by lambs did not alter growth performance variables but changed the activity/concentration of blood, ruminal environment, and meat markers. Among our primary conclusions, we highlight the intestinal and hepatic antioxidant activity associated with regulating ATP in the phosphorylation process. Furthermore, TB consumption increases the proportion of unsaturated FA in meat and reduces SFAs.

Authors' Contributions

Santos S. A. I, Da Silva A.S., and Kessler J.D. contributed to the design and implementation of the research for the analysis of results and preparation of the published work, explicitly writing the initial draft. Klein. B.; Wagner, R.; Silva L.E.L., Silva P.M. and Baldissera M.D. performed laboratory analysis. Brunetto, A. and Deolindo, G.L. participated in the experiment and collected samples and data.

Fund

This study was supported by the UDESC and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC) of Santa Catarina State; and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

Declarations

Ethics Approval

The project was approved by the ethics committee on the use of animals in research of UDESC (CEUA nº46844151222). The study followed the guidelines of the Brazilian Council for Animal Experimentation, Brazil.

Consent for Publication

All authors have consented to the publication and presentation of the data in this article.

Conflicts of Interests

The authors declare that they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

References

[1] Araujo, G., Terré, M., Mereu, A., Ipharraguerre, I.R. and Bach, A. (2016) Effects of Supplementing a Milk Replacer with Sodium Butyrate or Tributyrin on Performance and Metabolism of Holstein Calves. *Animal Production Science*, **56**, 1834-1841. https://doi.org/10.1071/an14930

[2] den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D. and Bakker, B.M. (2013) The Role of Short-Chain Fatty Acids in the Interplay between Diet, Gut Microbiota, and Host Energy Metabolism. *Journal of Lipid Research*, **54**, 2325-2340. https://doi.org/10.1194/jlr.r036012

[3] Bedford, A. and Gong, J. (2018) Implications of Butyrate and Its Derivatives for Gut Health and Animal Production. *Animal Nutrition*, **4**, 151-159. https://doi.org/10.1016/j.aninu.2017.08.010

[4] Alarcon, P., Manosalva, C., Carretta, M.D., Hidalgo, A.I., Figueroa, C.D., Taubert, A., *et al*. (2018) Fatty and Hydroxycarboxylic Acid Receptors: The Missing Link of Immune Response and Metabolism in Cattle. *Veterinary Immunology and Immunopathology*, **201**, 77-87. https://doi.org/10.1016/j.vetimm.2018.05.009

[5] Ren, Q., Xuan, J., Wang, L., Zhan, Q., Yin, D., Hu, Z., *et al*. (2018) Effects of Tributyrin Supplementation on Ruminal Microbial Protein Yield, Fermentation Characteristics and Nutrients Degradability in Adult Small Tail Ewes. *Animal Science Journal*, **89**, 1271-1279. https://doi.org/10.1111/asj.13033

[6] Carretta, M.D., Hidalgo, A.I., Burgos, J., Opazo, L., Castro, L., Hidalgo, M.A., *et al*.

(2016) Butyric Acid Stimulates Bovine Neutrophil Functions and Potentiates the Effect of Platelet Activating Factor. *Veterinary Immunology and Immunopathology*, **176**, 18-27. https://doi.org/10.1016/j.vetimm.2016.05.002

[7] Masmeijer, C., Rogge, T., van Leenen, K., De Cremer, L., Deprez, P., Cox, E., *et al.* (2020) Effects of Glycerol-Esters of Saturated Short- and Medium Chain Fatty Acids on Immune, Health and Growth Variables in Veal Calves. *Preventive Veterinary Medicine*, **178**, Article ID: 104983. https://doi.org/10.1016/j.prevetmed.2020.104983

[8] Liu, W., La, A.L.T.Z., Evans, A., Gao, S., Yu, Z., Bu, D., *et al.* (2021) Supplementation with Sodium Butyrate Improves Growth and Antioxidant Function in Dairy Calves before Weaning. *Journal of Animal Science and Biotechnology*, **12**, Article No. 2. https://doi.org/10.1186/s40104-020-00521-7

[9] Giacomini, P., Braga, F., Araujo, R., Cruz-Polycarpo, V. and Polycarpo, G. (2022) Meta-Analysis of Butyric Acid: A Performance-Enhancing Additive to Replace Antibiotics for Broiler Chickens. *Brazilian Journal of Poultry Science*, **24**, Article 1463. https://doi.org/10.1590/1806-9061-2021-1463

[10] Sotira, S., Dell'Anno, M., Caprarulo, V., Hejna, M., Pirrone, F., Callegari, M.L., *et al.* (2020) Effects of Tributyrin Supplementation on Growth Performance, Insulin, Blood Metabolites and Gut Microbiota in Weaned Piglets. *Animals*, **10**, Article 726. https://doi.org/10.3390/ani10040726

[11] Kovanda, L., Zhang, W., Wei, X., Luo, J., Wu, X., Atwill, E.R., *et al.* (2019) *In Vitro* Antimicrobial Activities of Organic Acids and Their Derivatives on Several Species of Gram-Negative and Gram-Positive Bacteria. *Molecules*, **24**, Article 3770. https://doi.org/10.3390/molecules24203770

[12] Liang, H., Ji, K., Ge, X., Xi, B., Ren, M., Zhang, L., *et al.* (2021) Tributyrin Plays an Important Role in Regulating the Growth and Health Status of Juvenile Blunt Snout Bream (*Megalobrama amblycephala*), as Evidenced by Pathological Examination. *Frontiers in Immunology*, **12**, Article 652294. https://doi.org/10.3389/fimmu.2021.652294

[13] Palma, M., Magnoni, L.J., Morais, S. and Viegas, I. (2022) Tributyrin Supplementation in Fish and Crustacean Nutrition: A Review. *Reviews in Aquaculture*, **15**, 785-800. https://doi.org/10.1111/raq.12759

[14] He, Z., Liu, N., Cai, Y., Yang, N., Li, G., Xiao, Y., *et al.* (2022) Effect of Tributyrin on Growth Performance and Pathway by Which Tributyrin Regulates Oligopeptide Transporter 1 in Juvenile Grass Carp (*Ctenopharyngodon idellus*). *Animals*, **12**, Article 2498. https://doi.org/10.3390/ani12192498

[15] Watanabe, D.H., Górka, P., Santin, E., Leclerc, H. and Penner, G.B. (2021) PSXIII-11 Evaluating Different Doses of Rumen-Protected or Nonprotected Tributyrin on Performance of Feedlot Lambs Fed a Moderate or Low-Forage Diet. *Journal of Animal Science*, **99**, 464-464. https://doi.org/10.1093/jas/skab235.823

[16] Dirksen, G. and Breitner, W. (1993) A New Quick-Test for Semiquantitative Determination of *β*-Hydroxybutyric Acid in Bovine Milk. *Journal of Veterinary Medicine Series A*, **40**, 779-784. https://doi.org/10.1111/j.1439-0442.1993.tb00694.x

[17] Baldissera, M.D., Souza, C.F., Júnior, G.B., Verdi, C.M., Moreira, K.L.S., da Rocha, M.I.U.M., *et al.* (2017) Aeromonas Caviae Alters the Cytosolic and Mitochondrial Creatine Kinase Activities in Experimentally Infected Silver Catfish: Impairment on Renal Bioenergetics. *Microbial Pathogenesis*, **110**, 439-443. https://doi.org/10.1016/j.micpath.2017.07.031

[18] Janssen, E. (2000) Adenylate Kinase 1 Gene Deletion Disrupts Muscle Energetic Economy Despite Metabolic Rearrangement. *The EMBO Journal*, **19**, 6371-6381. https://doi.org/10.1093/emboj/19.23.6371

[19] Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) Glutathione S-Transferases: The First Enzymatic Step in Mercapturic Acid Formation. *Journal of Biological Chemistry*, **249**, 7130-7139. https://doi.org/10.1016/s0021-9258(19)42083-8

[20] Beutler, E. (1984) Superoxide Dismutase. Red Cell Metabolism. A Manual of Biochemica Methods. Grune & Stratton.

[21] Bligh, E.G. and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification. *Canadian Journal of Biochemistry and Physiology*, **37**, 911-917. https://doi.org/10.1139/o59-099

[22] Hartman, L. and Lago, R.C.A. (1973) Rapid Preparation of Fatty Acid Methyl Esters. *Laboratory Practice*, **22**, 475-494

[23] Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R. and Van Immerseel, F. (2010) From the Gut to the Peripheral Tissues: The Multiple Effects of Butyrate. *Nutrition Research Reviews*, **23**, 366-384. https://doi.org/10.1017/s0954422410000247

[24] Sakata, T. and Tamate, H. (1978) Rumen Epithelial Cell Proliferation Accelerated by Rapid Increase in Intraruminal Butyrate. *Journal of Dairy Science*, **61**, 1109-1113. https://doi.org/10.3168/jds.s0022-0302(78)83694-7

[25] Dell'Anno M., Caprarulo V., Reggi S., Luisa Callegari M., Hejna M. and Rossi L. (2021) Tributyrin as Feed Supplement for Young Animals in Italian. *Journal of Animal Science*, **20**, 143-144

[26] Górka, P., Kowalski, Z.M., Pietrzak, P., Kotunia, A., Jagusiak, W., Holst, J.J., *et al*. (2011) Effect of Method of Delivery of Sodium Butyrate on Rumen Development in Newborn Calves. *Journal of Dairy Science*, **94**, 5578-5588. https://doi.org/10.3168/jds.2011-4166

[27] Murayama, K., Fukui, T., Kushibiki, S., Sakamoto, K., Inouchi, K. and Sugino, T. (2023) Effects of Medium-Chain Fatty Acids and Tributyrin Supplementation in Milk Replacers on Growth Performance, Blood Metabolites, and Hormone Concentrations in Holstein Dairy Calves. *Journal of Dairy Science*, **106**, 4599-4607. https://doi.org/10.3168/jds.2022-22957

[28] Wu, D., Zhang, Z., Shao, K., Wang, X., Huang, F., Qi, J., *et al*. (2023) Effects of Sodium Butyrate Supplementation in Milk on the Growth Performance and Intestinal Microbiota of Preweaning Holstein Calves. *Animals*, **13**, Article 2069. https://doi.org/10.3390/ani13132069

[29] Li, Y., Wang, H., Zhang, Y., Li, X., Jiang, X. and Ding, H. (2022) 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*, **12**, Article 1309. https://doi.org/10.3390/ani12101309

[30] Corrêa-Oliveira, R., Fachi, J.L., Vieira, A., Sato, F.T. and Vinolo, M.A.R. (2016) Regulation of Immune Cell Function by Short-Chain Fatty Acids. *Clinical & Translational Immunology*, **5**, e73. https://doi.org/10.1038/cti.2016.17

[31] Schlattner, U., Tokarska-Schlattner, M. and Wallimann, T. (2006) Mitochondrial Creatine Kinase in Human Health and Disease. *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, **1762**, 164-180. https://doi.org/10.1016/j.bbadis.2005.09.004

[32] Panayiotou, C., Solaroli, N. and Karlsson, A. (2014) The Many Isoforms of Human Adenylate Kinases. *The International Journal of Biochemistry & Cell Biology*, **49**, 75-83. https://doi.org/10.1016/j.biocel.2014.01.014

[33] Manso, T., Gallardo, B. and Guerra-Rivas, C. (2016) Modifying Milk and Meat Fat Quality through Feed Changes. *Small Ruminant Research*, **142**, 31-37. https://doi.org/10.1016/j.smallrumres.2016.03.003

[34] Alshamiry, F.A., Alharthi, A.S., Al-Baadani, H.H., Aljumaah, R.S. and Alhidary, I.A. (2023) Growth Rates, Carcass Traits, Meat Yield, and Fatty Acid Composition in Growing Lambs under Different Feeding Regimes. *Life*, **13**, Article 409. https://doi.org/10.3390/life13020409

[35] Calder, P.C. (2014) Very Long Chain ω-3 (n-3) Fatty Acids and Human Health. *European Journal of Lipid Science and Technology*, **116**, 1280-1300. https://doi.org/10.1002/ejlt.201400025

Appendix

Supplementary Material 1. Standardization of methods for measuring short-chain fatty acids.

	Acetic acid	Propionic acid	Butyric acid	Isovaleric acid
R^2	0.9998	0.9996	0.9998	0.9998
Equation	y = 0.011x − 0.0027	y = 0.0193x − 0.0121	y = 0.0268x − 0.0118	y = 0.034 − 0.0051
Linear range (mmol·L^{-1})*	2.22 - 132.99	1.72 - 102.95	1.35 - 81.15	0.57 - 18.30
LOD (mmol·L^{-1})	1.11	0.86	0.68	0.29
LOQ (mmol·L^{-1})	2.22	1.72	1.35	0.57
Accuracy	96.44	95.31	92.84	93.56
Repeatability (RSD)	3.31	2.54	2.14	1.80

The LR (linear range), LOD (limit of detection), and LOQ (limit of quantification) were expressed as mmol SCFA per L of rumen fluid.