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Effects of tributyrin supplementation on short-chain fatty acid concentration, fibrolytic enzyme activity, nutrient digestibility and methanogenesis in adult Small Tail ewes

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Abstract

In vivo and *in vitro* trials were conducted to assess the effects of tributyrin (TB) supplementation on short-chain fatty acid (SFCA) concentrations, fibrolytic enzyme activity, nutrient digestibility and methanogenesis in adult sheep. Nine 12-month-old ruminally cannulated Small Tail ewes (initial body weight 55 ± 5.0 kg) without pregnancy were used for the *in vitro* trial. *In vitro* substrate made to offer TB at 0, 2, 4, 6 and 8 g/kg on a dry matter (DM) basis was incubated by ruminal microbes for 72 h at 39°C. Forty-five adult Small Tail ewes used for the *in vivo* trial were randomly assigned to five treatments with nine animals each for an 18-d period according to body weight (55 ± 5.0 kg). Total mixed ration fed to ewes was also used to offer TB at 0, 2, 4, 6 and 8 g/kg on a DM basis. The *in vitro* trial showed that TB supplementation linearly increased apparent digestibility of DM, crude protein, neutral detergent fibre and acid detergent fibre, and enhanced gas production and methane emissions. The *in vivo* trial showed that TB supplementation decreased DM intake, but enhanced ruminal fermentation efficiency. Both *in vitro* and *in vivo* trials showed that TB supplementation enhanced total SFCA concentrations and carboxymethyl cellulase activity. The results indicate that TB supplementation might exert advantage effects on rumen microbial metabolism, despite having an enhancing effect on methanogenesis.

Introduction

Butyric acid plays a key role in maintaining gut health in animals, as a major source of energy to colonic mucosa and an important regulator of gene expression, differentiation, inflammation and apoptosis in host cells (Pajak *et al.,* 2007; Hamer *et al.,* 2008). Butyrate supplementation improved feed efficiency, digestibility of nutrients and growth in young calves (Guilloteau *et al.,* 2009). During absorption in the rumen, around 0.9 of butyric acid is oxidized to ketone bodies (Britton and Krehbil, 1993). Development of the ketogenic capacity of ruminal epithelium occurs as the animal ages and genes encoding the enzymes controlling ketogenesis are expressed independently of intra-ruminal butyric acid concentration (Lane *et al.,* 2002). Thus, butyrate supplementation should be provided to young ruminants after birth when the ruminal butyrate concentration is quite low: in adult ruminants, butyric acid is one of the main products of ruminal fermentation, and its proportion in total fermentation acids varies between 0.05 and >0.2 (Aschenbach *et al.,* 2011). However, the effects of butyrate depend not only on animals' age but also on the experimental model (*in vivo* or *in vitro*) and supplementation doses used (Guilloteau *et al.,* 2010).

Tributyrin (TB), composed of butyric acid and glycerol, is a triglyceride naturally present in butter. In the rumen, TB can be metabolized into three free butyric acid molecules by microbes. Compared with sodium butyrate, TB has more favourable pharmacokinetics because of its more potent and direct effect on cells (Chen and Breitman, 1994). Although TB has been used as a feed additive to assess its effects on performance and metabolism in Holstein calves (Araujo *et al.,* 2016), research on its positive effects on metabolism in adult ruminants is still quite limited. Thus, the current study aimed to evaluate the positive effects of TB supplementation in adult Small Tail ewes through both *in vitro* and *in vivo* trials, particularly to assess the effects on short-chain fatty acid (SCFA) concentrations, fibrolytic enzyme activities, nutrient degradation and methane emissions.

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Materials and methods

The *in vitro* trial was conducted at the Key Laboratory of College of Animal Science, Anhui Science and Technology University (Fengyang, China), while the *in vivo* trial was conducted at the Experimental Station of Anhui Province Modern Agriculture Technology System in Cattle and Sheep (Bengbu, China).

In vitro *trial*

Nine 12-month-old, non-pregnant, ruminally cannulated Small Tail ewes (initial body weight 55 ± 5.0 kg, mean ± standard deviation) were confined in a 27-m^2 concrete-floor pen, in which 12 feed bunks and two watering points were provided for *ad libitum* consumption. Ewes were fed twice daily (at 07.00 and 19.00 h) *ad libitum* with a total mixed ration (TMR) consisting of 256 g maize meal/kg, 64 g wheat bran/kg, 51 g soybean meal/kg, 29 g premix/kg, 400 g ensiled maize stover/kg and 200 g peanut straw/kg.

A completely randomized design was applied to five runs of *in vitro* batch cultures, and 0.5 g experimental substrate (Table 1) was weighed into 90 bottles/run with 18 bottles for each treatment, to offer TB (Sigma Aldrich, St. Louis, MO, USA) at 0 (control), 2, 4, 6 and 8 g/kg on a dry matter (DM) basis. In each run, 50 ml freshly prepared buffer solution (pH 6.85, Menke and Steingass, 1988) were added to the bottles. Rumen fluids collected from nine ewes were filtered through two layers of muslin and mixed in equal volume ratios, and then 25 ml rumen fluid were added to the bottles to serve as a donor of mixed rumen microorganisms. All bottles were purged with anaerobic nitrogen gas (N_2) for 5 s, sealed with butyl rubber stoppers and Hungate screw caps, and then incubated at 39 °C for 72 h. Three fermentations without substrate and TB supplemented were used as blanks in each run. If necessary, through analysing the concentrations of microbial protein and total SCFA in the blanks, the variation between runs caused by different rumen fluid inoculum in different periods could be checked.

After 72 h of incubation, head-space gas pressure was measured by using a pressure transducer to estimate the cumulative gas volume, and production of hydrogen (H_2), methane (CH_4) and carbon dioxide (CO_2) were analysed. All culture fluids were filtered with nylon bags (48.0 μm pore size) and then 10 ml filtered fluids were prepared for analysis of pH, SCFA concentration and fibrolytic enzyme activities. The residual contents of the substrate were collected for analysis of DM, crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF).

In vivo *trial*

Forty-five 12-month-old Small Tail ewes were assigned randomly to five groups with nine animals each (live weight 55 ± 5.0 kg, mean ± standard deviation). The ewes were kept in individual cages (1.5 × 1.5 m^2) on a perforated wooden floor without litter. During the experiment, water was available *ad libitum*. The ewes were fed individually twice daily at 07.00 and 19.00 h with TMR (Table 1), which was formulated at a constant concentrate-to-forage ratio (40:60). The TMR was supplemented with TB at 0, 2, 4, 6 and 8 g/kg on a DM basis, according to previous research (Araujo *et al.*, 2016) in which TB was supplemented at a level of 3 g/kg DM in the calf ration. The offered and refused rations were recorded to assess the effect of TB supplementation on DM intake.

The *in vivo* trial lasted 18 days, including 15 days to allow diet adaptation and the last 3 days to collect 12 rumen fluid samples: rumen fluid was sampled four times on each of these days at 6-h intervals. The sampling times were moved forward by 2 h each day compared with the previous day, so that after 3 days there were samples for every 2-h interval in 24 h for each ewe. Samples were collected from the rumen according to the methods of oral tube collection described by Sorensen and Schambye (1955). The rumen fluids collected at different sampling time points were pooled in equal portion, hand-mixed thoroughly and filtered through four layers of muslin. After pH measurement, the rumen fluids were centrifuged at 1000***g*** for 30 min at 4 °C, and the supernatant was separated and immediately stored at −20 °C for analysis of SCFA concentrations and fibrolytic enzyme activities.

Table 1. Ingredient and chemical composition of the basal diet

Item	g/kg DM
Ingredient	
Maize	256.0
Wheat bran	64.0
Soybean meal	51.2
Ensiled maize stover	400.0
Peanut straw	200.0
Premix[a]	28.8
Analysed chemical composition of feed	
Metabolizable energy[b] (MJ/kg DM)	10.2
Crude protein (g/kg DM)	90.1
Ether extract (g/kg DM)	52.1
Ash (g/kg DM)	50.9
Calcium (g/kg DM)	11.9
Total phosphorus (g/kg DM)	4.2
Neutral detergent fibre (g/kg DM)	397.3
Acid detergent fibre (g/kg DM)	247.4
Non-fibre carbohydrate[c] (g/kg DM)	409.6

DM, dry matter.
[a]Per kilogram of premix contained 1 54 400 international unit (IU) of Vitamin A, 94 000 IU of Vitamin D_3, 3 38 200 IU of Vitamin E, 120.0 mg iodine, 280.0 mg copper, 2240.0 mg iron, 1740.0 mg manganese, 1370.0 mg zinc, 60.0 mg selenium, 16.8 mg cobalt, 50.0 mg of rumen protection of Met and Lys.
[b]Metabolizable energy was estimated according to NRC (2001).
[c]Non-fibre carbohydrate (g/kg) = 1000 − (NDF + CP + EE + Ash).

Chemical analysis

Residual content analysis

Following AOAC (2012) procedures, *in vitro* residual contents and *in vivo* feed offered and refused were analysed for DM (ID 930.15) and CP (ID 984.13). Both NDF and ADF were analysed (Van Soest *et al.*, 1991) with heat-stable α-amylase (Sigma no. A3306; Sigma Chemical Co., St. Louis, MO, USA) and corrected for residual ash content. *In vitro* apparent degradations of DM, CP, NDF and ADF were calculated from Eqn (1):

$$\text{Apparent degradation (DM basis)} = \frac{\text{initial content} - \text{residual content}}{\text{initial content}} \quad (1)$$

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Table 2. Effects of tributyrin supplementation on *in vitro* fermentation characteristics at 72 h

	Tributyrin supplementation (g/kg DM)						*P*-value[a]		
Item	0	2	4	6	8	S.E.M.	Contrast	Linear	Quadratic
pH	6.6	6.6	6.5	6.4	6.4	0.04	0.063	0.004	0.860
Total SCFA[b] (mM)	83	86	90	93	98	1.8	<0.001	<0.001	0.566
Acetate (mM)	62	64	66	68	73	1.7	0.005	<0.001	0.724
Propionate (mM)	11.4	12.2	13.6	13.5	13.5	0.60	0.014	0.009	0.5517
Butyrate (mM)	4.4	4.6	4.9	4.9	5.2	0.19	0.030	0.007	0.526
Valerate (mM)	2.5	2.6	2.9	3.0	3.0	0.17	0.048	0.015	0.937
Branched chain SCFA[c] (mM)	2.3	2.5	2.5	2.7	2.6	0.64	<0.001	<0.001	0.209
NGR[d]	5.3	5.0	4.8	4.9	5.2	0.25	0.245	0.642	0.910
FE[e]	0.7	0.7	0.7	0.7	0.7	0.01	0.344	0.812	0.654

DM, dry matter; SCFA, short-chain fatty acids; NGR, ratio of non-glucogenic to glucogenic SCFAs; FE, fermentation efficiency.
[a]Contrast, supplemental effect of tributyrin; Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.
[b]Total SCFA = acetate + propionate + butyrate + valerate + branched chain SCFA.
[c]Branched chain SCFA including iso-butyrate and iso-valerate.
[d]NGR calculated according to Orskov (1975): NGR = (Acetate + 2 × Butyrate + Valerate)/(Propionate + Valerate).
[e]FE = (0.622 × Acetate + 1.092 × Propionate + 1.56 × Butyrate)/(Acetate + Propionate + 2 × Butyrate) (Abdl-Rahman, 2010).

Rumen fluid pH and short-chain fatty acid analysis

The filtered rumen fluid pH was measured immediately using a pH meter (Model pHs-29A, Jingke Leici Co. Ltd, Shanghai, China). After thawing at room temperature, the rumen fluid samples (1 ml) were mixed with 0.3 ml of 25% (w/v) meta-phosphoric acid solution for 30 min and then centrifuged at 15 000***g*** for 10 min at 4 °C. Following the method of Yang *et al.* (2005), concentrations of acetate, propionate, butyrate, valerate and branched chain SCFAs including iso-butyrate and iso-valerate in the supernatants were measured using a gas chromatograph (GC522, Wufeng instruments, Shanghai, China).

Fibrolytic enzyme assays

Reducing sugar (expressed as glucose) release was determined as described by MacKenzie and Bilous (1982) with glucose used as the standard, and one unit of enzyme activity was defined as the amount of enzyme that released 1 μM of reducing sugar/min in 1 ml fluid.

Gas generation assay

A pressure transducer interfaced with a computer was used to measure accumulated head-space gas pressure; the values could be entered directly into the computer and used to estimate the cumulative gas volumes. Before the onset of the *in vitro* trial, a standard curve was made to describe the quantitative relationship between gas volumes and pressures in bottles with 75 ml buffer solutions at 39 °C. The production of H_2, CO_2 and CH_4 were measured following the method described by Zhang and Yang (2011).

Statistical analysis

In vitro and *in vivo* data were analysed using PROC MIXED model of SAS 9.4 (Statistical Analysis for Windows, SAS Institute Inc., Cary, NC, USA). Linear and quadratic effects of treatments indicated by orthogonal contrasts were used to evaluate effects of TB supplementation. Duncan's multiple range test was conducted to determine the significance level of the particular comparison between treatment means. Differences were considered significant at $P \leqslant 0.05$. The model including random and fixed effects was as follows:

$$Y_{ij} = m + R_i + T_j + e_{ij} \quad (2)$$

where Y_{ij} is the dependent variable, μ is the overall mean, R_i is the random effect (for *in vitro* random effect of run, $i = 5$; for *in vivo* random effect of ewe, $i = 9$), T_j is the fixed effect of TB ($j = 0$, 2, 4, 6, 8) and ε_{ij} is the error term.

Results

Effects of substrate supplementation with tributyrin on in vitro *fermentation, nutrient digestibility and gas production characteristics*

Substrate supplementation of TB (Table 2) linearly decreased *in vitro* pH of the filtered rumen fluid ($P = 0.005$) and increased concentrations of total SCFA ($P < 0.001$), acetate ($P < 0.001$), propionate ($P < 0.001$), butyrate ($P = 0.007$), valerate ($P = 0.015$) and branched chain SCFA ($P < 0.001$). However, TB supplementation had no effect on *in vitro* ratio of non-glucogenic to glucogenic SCFAs (NGR) and fermentation efficiency (FE).

As shown in Table 3, TB supplementation linearly increased *in vitro* apparent digestibility of DM ($P < 0.001$), CP ($P < 0.001$), NDF ($P < 0.001$) and ADF ($P = 0.001$), as well as *in vitro* gas production ($P = 0.017$) (Table 4). It decreased production of H_2 ($P < 0.001$) and CO_2 ($P = 0.017$), but increased CH_4 production ($P < 0.001$).

Effects of tributyrin supplementation on both in vitro *and* in vivo *fibrolytic enzyme activities*

Substrate supplementation of TB (Table 5) quadratically increased *in vitro* carboxymethyl cellulase (CMCase) activity ($P = 0.004$). Feed supplementation of TB linearly increased activity of xylanase ($P < 0.001$) and CMCase ($P = 0.017$). Compared with the control, TB supplementation tended to increase *in vivo* avicelase activity ($P = 0.099$).

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Table 3. Effects of tributyrin supplementation on *in vitro* apparent degradations of dry matter (DM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) at 72 h

	Tributyrin supplementation (g/kg DM)						*P*-value[a]		
Item	0	2	4	6	8	SEM	Contrast	Linear	Quadratic
DM (g/kg)	276	301	330	346	355	15.4	0.003	<0.001	0.855
CP (g/kg DM)	347	381	425	458	516	18.8	<0.001	<0.001	0.717
NDF (g/kg DM)	313	323	363	380	395	17.0	0.012	<0.001	0.602
ADF (g/kg DM)	282	303	353	353	370	18.9	0.008	0.001	0.367

[a]Contrast, supplemental effect of tributyrin; Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.

Table 4. Effects of tributyrin supplementation on *in vitro* gas production characteristics at 72 h

	Tributyrin supplementation (g/kg DM)						*P*-value[a]		
Item	0	2	4	6	8	SEM	Contrast	Linear	Quadratic
GP_{72} (ml/g DM)	27.8	28.9	29.7	29.7	31.4	0.98	0.067	0.017	0.724
H_2 (ml/100 ml)	0.9	0.8	0.6	0.6	0.5	0.12	0.001	<0.001	0.760
CH_4 (ml/100 ml)	20.1	21.2	21.5	22.1	25.2	0.54	0.003	<0.001	0.897
CO_2 (ml/100 ml)	78.8	77.8	77.8	77.1	74.2	0.55	0.067	0.017	0.724

DM, dry matter; GP_{72}, gas production at 72 h; H_2, hydrogen gas; CH_4, methane; CO_2, carbon dioxide.
[a]Contrast, supplemental effect of tributyrin; Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.

Effects of feed supplementation of tributyrin on dry matter intake and in vivo *ruminal fermentation characteristics*

Feed supplementation of TB (Table 6) decreased DM intake ($P < 0.001$) and ruminal pH ($P < 0.001$) but linearly increased concentrations of total ($P < 0.001$) and most individual SCFA ($P \leqslant 0.01$) except valerate ($P = 0.123$). Feed supplementation of TB had no effect on *in vivo* NGR but enhanced ruminal FE ($P = 0.009$).

Discussion

Butyrate is a major microbial fermentation product in the rumen. In the current *in vitro* and *in vivo* trials, the same supplementary dosages of TB were used to avoid possible confounding effects of butyrate on fermentation characteristics.

Feed supplementation of TB decreased *in vivo* DM intake in ewes compared with the control group, possibly because: (i) TB used as a feed additive may modify the taste of feed and cause greater amounts of refusals, despite its stability and low odour; and (ii) TB might act as an energy source by undergoing β oxidation to acetyl-coenzyme A (acetyl-CoA) and generating adenosine triphosphate (ATP) for TB-supplemented ewes (Donohoe *et al.*, 2011).

In both trials, decreased pH values might be due to the increased total SCFA concentration in the rumen and culture fluids (Burrin and Britton, 1986). Alternatively, SCFAs also play an important role as the major energy sources for ruminants. In the current study, both *in vitro* and *in vivo* total SCFA concentrations were positively related to TB supplementation, which might be attributed to the stimulating effects of TB on the concentrations of individual fatty acids. Li *et al.* (2012) found that exogenous butyrate stimulated native butyrate-producing bacteria population such as *Butyrivibrio* and *Pseudobutyrivibrio*, which are probably the predominant butyrate producers in the rumen microbial ecosystem. In addition, growth of ruminal *Bacteroidetes, Firmicutes* and *Fibrobacteres*, which are essential in converting carbohydrates to SCFAs, were also increased by exogenous butyrate (Li *et al.*, 2012).

In the current study, increased butyrate concentration might be related to the hydrolysis of TB by rumen microorganisms. Alternatively, butyrate synthesis by rumen microorganisms may be related to the utilization of acetate or compounds giving rise to acetyl-CoA such as pyruvate (Barker, 1961).

Unlike acetic, propionic and butyric acids, branched chain SCFAs including iso-butyrate and iso-valerate are produced by the breakdown of protein and are essential for synthesis of cellular constituents by ruminal bacteria (Allison, 1969). Elastase II activity related to protein digestibility in calves was enhanced by sodium butyrate supplementation (Guilloteau *et al.*, 2009), which is beneficial to generate branched chain SCFA.

The optimal value of NGR in the rumen is about 3.5, and a value lower than 3.5 indicates more efficient utilization of SCFA for gluconeogenesis (Abdl-Rahman, 2010). In the current study, TB supplementation had no effect on the fermentation pattern in ewes indicated by the fairly constant NGR, which is consistent with previous research (Kowalski *et al.*, 2015). Likewise, Malhi *et al.* (2013) reported that the fermentation pattern was not affected in goats infused intra-ruminally with 0.3 g/kg body weight of butyrate/day.

Xylanase, CMCase and avicelase are the primary fibrolytic enzymes for the hydrolysis of dietary carbohydrates in the rumen (Santra *et al.*, 2007). Ruminal microorganisms such as bacteria, protozoa and phycomycete fungi may provide a wide range of fibrolytic enzymes to degrade feed fibre (Chen *et al.*, 2008). Thus, the enhanced fibrolytic enzyme activity in the current study might be attributed to the stimulating effects of TB on butyrate-producing

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Table 5. Effects of tributyrin supplementation on both *in vitro* and *in vivo* fibrolytic enzyme activities

	Tributyrin supplementation (g/kg DM)						*P*-value[a]		
Item	0	2	4	6	8	SEM	Contrast	Linear	Quadratic
In vitro fibrolytic enzyme activity									
Xylanase (mU)	128	130	158	140	148	11.3	0.188	0.155	0.128
CMCase (mU)	28	29	35	38	33	2.2	0.017	0.770	0.004
Avicelase (mU)	4.2	5.4	5.3	4.6	4.9	0.70	0.259	0.771	0.819
In vivo fibrolytic enzyme activity									
Xylanase (mU)	80	89	90	112	120	9.6	0.035	<0.001	0.431
CMCase (mU)	37	47	49	56	54	5.8	0.200	0.017	0.528
Avicelase (mU)	11.0	12.9	12.5	12.1	12.2	0.75	0.099	0.519	0.817

DM, dry matter; CMCase, carboxymethyl cellulase.
[a]Contrast, supplemental effect of tributyrin; Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.

Table 6. Effects of tributyrin supplementation on dry mater intake (DMI) and *in vivo* ruminal fermentation characteristics in adult ewes

	Tributyrin supplementation (g/kg DM)						*P*-value[a]		
Item	0	2	4	6	8	SEM	Contrast	Linear	Quadratic
DMI (kg/day)	1.0	1.0	0.9	1.0	0.9	0.01	<0.001	<0.001	<0.001
pH	6.8	6.7	6.5	6.5	6.5	0.03	<0.001	<0.001	0.081
Total SCFA[b] (mM)	97	101	103	102	105	1.6	0.001	<0.001	0.595
Acetate (mM)	79	82	83	83	85	1.4	0.005	0.003	0.606
Propionate (mM)	10.7	11.3	11.8	11.8	12.2	0.30	0.002	<0.001	0.585
Butyrate (mM)	4.6	4.7	4.8	5.0	5.3	0.10	<0.001	<0.001	0.911
Valerate (mM)	1.0	1.0	1.0	1.0	1.0	0.01	0.548	0.123	0.299
Branched chain SCFA[c] (mM)	1.9	1.9	1.9	1.9	2.0	0.02	0.211	0.010	0.999
NGR[d]	7.6	7.5	7.4	7.4	7.6	0.13	0.261	0.711	0.719
FE[e]	0.7	0.7	0.7	0.7	0.7	0.01	0.063	0.009	0.814

DM, dry matter; SCFA, short chain fatty acids; NGR, ratio of non-glucogenic to glucogenic SCFAs; FE, fermentation efficiency.
[a]Contrast, supplemental effect of tributyrin; Linear, linear effect of tributyrin; Quadratic, quadratic effect of tributyrin.
[b]Total SCFA = acetate + propionate + butyrate + valerate + branched chain SCFA.
[c]Branched chain SCFA including iso-butyrate and iso-valerate.
[d]NGR calculated according to Orskov (1975): NGR = (Acetate + 2 × Butyrate + Valerate)/(Propionate + Valerate).
[e]FE = (0.622 × Acetate + 1.092 × Propionate + 1.56 × Butyrate)/(Acetate + Propionate + 2 × Butyrate) (Abdl-Rahman, 2010).

bacteria (Guilloteau *et al.*, 2010; Li *et al.*, 2012), which is responsible for fibre digestion and utilization in the rumen (Mrazek *et al.*, 2006). In addition to the enhanced fibrolytic enzyme activities, *in vitro* apparent nutrient digestibility of substrate was increased by TB supplementation, which was consistent with a previous report by Huhtanen *et al.* (1993) that apparent digestibility of dietary DM, CP and NDF was enhanced by the increased ruminal supply of butyrate in dairy cows. Thus, the results of the current study indicate the positive effects of TB supplementation on nutrient utilization and fermentation efficiency.

Methane production indicates an energy loss to ruminants. In the present study, increased CH_4 production by TB supplementation is probably related to the reduced H_2 and CO_2, which were probably used by methanogenic bacteria to generate methane. Wang *et al.* (2009) demonstrated that even though butyric acid concentration was high, up to 1800 mg/l, no significant inhibition effect was observed on the activity of methanogenic bacteria. The increased methanogenesis by TB supplementation in the present study could have potentially negative effects on animal performance.

Conclusions

The current study demonstrated positive effects of TB supplementation on total and most individual SCFA concentrations, fibrolytic enzyme activities, as well as *in vitro* apparent nutrient digestibility and *in vivo* fermentation efficiency. These results suggest that TB supplementation might exert a positive influence on rumen microbial metabolism in adult ewes, despite having an enhancing effect on methane production.

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Ethical standards. This study was approved by the ethics committee of Anhui Science and Technology University (ECASTU-2015-P08).

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