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Effects of rumen-protected folic acid and betaine supplementation on growth performance, nutrient digestion, rumen fermentation and blood metabolites in Angus bulls

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Abstract

This study evaluated the effects of rumen-protected folic acid (RPFA) and betaine (BT) on growth performance, nutrient digestion and blood metabolites in bulls. Forty-eight Angus bulls were blocked by body weight and randomly assigned to four treatments in a 2 × 2 factorial design. BT of 0 or 0·6 g/kg DM was supplemented to diet without or with the addition of 6 mg/kg DM of folic acid from RPFA, respectively. Average daily gain increased by 25·2 and 6·29 % for addition of BT without RPFA and with RPFA, respectively. Digestibility and ruminal total volatile fatty acids of neutral-detergent fibre and acid-detergent fibre increased, feed conversion ratio and blood folate decreased with the addition of BT without RPFA, but these parameters were unchanged with BT addition in diet with RPFA. Digestibility of DM, organic matter and crude protein as well as acetate:propionate ratio increased with RPFA or BT addition. Ruminal ammonia-N decreased with RPFA addition. Activity of carboxymethyl cellulase, cellobiase, xylanase, pectinase and protease as well as population of total bacteria, protozoa, *Fibrobacter succinogenes* and *Ruminobacter amylophilus* increased with RPFA or BT addition. Laccase activity and total fungi, *Ruminococcus flavefaciens* and *Prevotella ruminicola* population increased with RPFA addition, whereas *Ruminococcus albus* population increased with BT addition. Blood glucose, total protein, albumin, growth hormone and insulin-like growth factor-1 increased with RPFA addition. Addition of RPFA or BT decreased blood homocysteine. The results indicated that addition of BT stimulated growth and nutrient digestion in bulls only when RPFA was not supplemented.

Key words: **Betaine: Bulls: Folic acid: Growth performance: Rumen fermentation**

Folic acid (FA) plays a major role in the synthesis of protein and DNA via mediating the transfer of one-carbon units[1,2]. The 5-methyl-tetrahydrofolate (THF) transfers its methyl group to homocysteine (Hcy) for the generation of methionine, 5,10-methylene-THF provides its CH_2 unit for the synthesis of thymidylate and 10-formyl-THF is used in the *de novo* synthesis of purine[1,2]. Studies indicated that the increase in milk protein yield with FA addition was associated with the stimulating effect of FA on DNA synthesis in dairy cows[3] and the improvement in growth performance following rumen-protected FA (RPFA) addition was companied with an up-regulated gene expression of the mammalian target of rapamycin signalling pathway in the liver of dairy calves[4]. Other studies found that dietary FA addition increased the relative abundance of ruminal cellulolytic bacteria in post-weaned calves[5] and the stimulating effect of FA on ruminal *Ruminococcus flavefaciens* growth was associated with its function of methyl transfer[6]. The data above indicated that dietary FA addition was required by both ruminal microbes and animal *per se*. Nevertheless, Santschi *et al.*[7] found that about 97 % of dietary supplemented FA would disappear in the rumen. Therefore, RPFA, which could provide FA both in the rumen and small intestine[4,8], should be used. Moreover, previous studies observed that addition of RPFA stimulated ruminal cellulolytic bacteria growth and nutrient digestion in steers[8] and increased average daily gain (ADG) in dairy calves[4].

Betaine (BT), a trimethyl derivative of glycine, is derived from choline oxidation and can be used as an important osmolyte and methyl donor in animals[2]. Studies in single-stomach

Abbreviations: ADF, acid-detergent fibre; ADG, average daily gain; BT, betaine; BW, body weight; CP, crude protein; DMI, DM intake; FA, folic acid; FCR, feed conversion ratio; Hcy, homocysteine; NDF, neutral-detergent fibre; OM, organic matter; RPFA, rumen-protected folic acid; THF, tetrahydrofolate; VFA, volatile fatty acids.

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animals reported that intestinal cell activity, digestive tract microbial abundance and nutrient digestibility increased with BT addition[2,9]. Wang *et al.*[10] found that rumen total volatile fatty acids (VFA) production and apparent total-tract nutrient digestibility increased with dietary BT addition in dairy cows. The data suggested that addition of BT might have a stimulating effect on ruminal microbial growth and enzymatic activity in ruminants. In addition, BT could provide methyl groups for the formation of 5-methyl-THF as well as for the regeneration of methionine from Hcy[2].

Considering the role of FA in transferring one-carbon units[2] and the property of BT as methyl donor[2] as well as the beneficial effects of FA and BT on rumen fermentation and nutrient digestion[8,10], we speculated that there might be an interaction between RPFA and BT on regulating growth, rumen fermentation and nutrient digestion in bulls. Therefore, this experiment was conducted to investigate the effects of dietary RPFA or/and BT addition on growth performance, nutrient digestion, rumen fermentation, enzymatic activity, microbiota and blood metabolites in Angus bulls.

Materials and methods

Animal welfare

Animal welfare, husbandry and experimental procedure were evaluated and authorised by the Animal Care and Ethics Committee of Shanxi Agriculture University (Taigu, Shanxi Province, China).

Angus bulls, experimental design and basal diet

This experiment was conducted from August 2018 to October 2018 at a commercial beef farm (Fanshi Tianhe Beef Farm). Forty-eight Angus bulls (363 (SD 10·5) days of age and 435 (SD 9·4) kg of body weight (BW)) were blocked by BW and randomly assigned to one of the four treatments with a 2 × 2 factorial design. Supplemental RPFA (0 mg/kg DM of FA (RPFA−) or 6 mg/kg DM of FA from RPFA (RPFA+)) and BT (0 g/kg DM of BT (BT−) or 0·6 g/kg DM of BT (BT+)) were mixed into the basal diet, respectively. The addition level of FA was based on the study of Wang *et al.*[8], in which ruminal total VFA concentration and nutrient degradability increased with dietary addition of 1·2 g/d of RPFA for each steer. Supplement of RPFA (20 g/kg of FA) was manufactured based on the method of Wang *et al.*[8]. The degradation rate of RPFA was determined by using four rumen and duodenum cannulated cows and was 0·23 and 0·67 in the rumen and in the small intestine, respectively. Three RPFA samples, six replicates and 5 g RPFA of each, were put into nylon bags and incubated in the rumen of each steer for 24 h. Three replicates of each sample collected from the rumen were put into the duodenum of steers and then collected from faeces. Residues were collected from the rumen, and faeces were washed in cold water for 3 min using a washing machine, dried at 55°C for 48 h in a forced air oven and determined DM and FA content. Supplement of BT (feed grade, 0·98 g/g; Shandong Gelande Biotechnology Co. Ltd) was purchased commercially and supplemented at the level of 0·6 g/kg DM according to the recommendation of the manufacture. The experiment lasted for 80 d with a 20-d adaptation period and then followed by a 60-d collection period. Bulls were housed in single pens (2·5 m × 3 m) and had free access to diet and clean water. The basal diet (Table 1) was formulated according to the National Research Council[11] and was offered to bulls twice daily at 07.00 and 19.00 hours.

Table 1. Ingredients and chemical composition of the basal diet*

	Contents (g/kg DM)
Ingredients	
Maize silage	370
Peanut vine	85
Oat hay	45
Maize grain, ground	279
Wheat bran	55
Soyabean meal	88
Rapeseed meal	25
Cottonseed cake	35
Calcium carbonate	5
Salt	5
Calcium phosphate	3
Mineral and vitamin premix†	5
Chemical composition	
Organic matter	932
Crude protein	135
Diethyl ether extract	28·0
Neutral-detergent fibre	392
Acid-detergent fibre	228
Non-fibre carbohydrate‡	376
Ca	7·6
P	4·1
Folate (mg/kg)	0·34

* Design values for ingredients and mean values (n 6) for chemical composition.
† Contained per kg premix: 1600 mg Cu, 8000 mg Mn, 7500 mg Zn, 1·20 mg iodine, 20 mg Co, 1·64 g vitamin A, 0·6 g vitamin D and 10 g vitamin E.
‡ Non-fibre carbohydrate, calculated by 1000 – crude protein – neutral-detergent fibre – fat – ash.

Sampling and measurements

During the sample collection period, DM intake (DMI) of each bull was measured daily and was calculated by the difference of feed offered and refused. Samples of feed offered and refused were collected every 5 d and stored at −20°C. Bulls were weighed on days 1, 30 and 60 before the 07.00 hours feeding. Acid-insoluble ash was used as an internal marker to determine total-tract apparent digestibility of DM, organic matter (OM), crude protein (CP), neutral-detergent fibre (NDF) and acid-detergent fibre (ADF)[12]. From days 54 to 57, about 250 g faecal samples were taken from the rectum of each bull at 06.00, 12.00, 18.00 and 24.00 hours and stored at −20°C. At the end of the sampling, feed, refusals and faeces were pooled by bull, dried at 55°C for 48 h and ground to pass through a 1 mm sieve with a cutter mill (110, Qingdao Ruixintai instrument Co. Ltd) for chemical analysis.

On days 58 and 59 of sample collection period, ruminal fluid of each bull was sampled by using an oral stomach tube at 06.30, 12.30, 18.30 and 00.30 hours. The first collected 150 ml of ruminal fluid was abandoned, and the next 100 ml was retained. After determining pH using a portable pH meter (PHB-4, Shanghai

Precision Scientific Instrument Co. Ltd), ruminal fluid was filtered through four layers of cheesecloth. One 5 ml of filtrate was mixed with 1 ml of 250 g/l meta-phosphoric acid for VFA determination; another 5 ml of filtrate was mixed with 1 ml of 20 g/l (w/v) H_2SO_4 for ammonia-N determination and kept at –20°C. Filtrate used for enzyme activity (15 ml) and microbial DNA (5 ml) determination was stored at –80°C.

On day 60 of sample collection period, blood samples of each bull were collected from the coccygeal vessel at 10.00 hours using 10 ml evacuated tubes (Jiancheng Biological Engineering Co. Ltd), centrifuged at 2500 ***g*** and 4°C for 10 min to separate serum and stored at –20°C.

Analytical methods

Samples of feed offered, feed refused and faeces were determined for DM (method 934.01), ash (method 942.05), N_2 (method 976.05), diethyl ether extract (method 973.18) and ADF (method 973.18) according to AOAC[13]. Content of OM was calculated by the difference between DM and crude ash. The NDF was measured based on the method of Van Soest *et al.*[14] with heat stable *α*-amylase and sodium sulphite used and expressed inclusive of residual ash. Acid-insoluble ash of faecal samples was measured based on the method of Van-Keulen & Young[12]. Folate in the basal diet was measured according to the method of Alaburda *et al.*[15]. Rumen VFA concentration was analysed by GC (Trace1300; Thermo Fisher Scientific Co. Ltd) with 2-ethylbutyric acid as an internal standard. Ammonia-N concentration was measured by a colorimetric spectrophotometer (UV2100, Shanghai Younike instrument Co. Ltd) according to AOAC[13]. Ruminal fluid was sonicated at 4°C for 10 min with a 20 s pulse rate and then centrifuged at 25 000 ***g*** and 4°C for 15 min to separate supernatant for measuring enzyme activity. Activity of carboxymethyl cellulase, xylanase, *α*-amylase, protease[16], cellobiase, pectinase[17] and laccase[18] was determined. Serum glucose, albumin, total protein, growth hormone, insulin-like growth factor-1, folate, Hcy and methionine was analysed by the Konelab TM auto analyzer (Thermo Fisher Scientific Oy) by using the corresponding ELISA test assay kit (Shanghai Meilian Biology Science & Technology Co. Ltd, China), respectively.

Microbial DNA extraction and real-time PCR

The homogenised rumen fluid of 1·2 ml was used for total microbial DNA isolation by using the repeated bead-beating plus column method[19]. The quality and quantity of microbial DNA were determined via agarose gel electrophoresis and NanoDrop 2000 Spectrophotometer (Thermo Scientific), respectively. The target microbes were total bacteria, total protozoa, total fungi, *Ruminococcus albus*, *Prevotella ruminicola*, *R. flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens* and *Ruminobacter amylophilus*. The sequences of all primer sets are shown in Table 2. The sample-derived standards of all target microbes were prepared from the treatment pool set of microbial DNA. A sample-derived DNA standard for every real-time PCR assay was generated by using the regular PCR. Subsequently, the PCR product was purified using a MiniBEST DNA Fragment Purification on Kit version 4.0 (Takara Biomedical Technology Co. Ltd) and quantified by a spectrophotometer. Copy number concentration of each sample-derived standard was evaluated according to the PCR product length and its mass concentration. The target microbial DNA was quantified by using serial 10-fold gradient dilutions from 10^1 to 10^8 DNA copies[20]. Amplification and detection of real-time PCR were carried out in a StepOne™ real-time PCR system (Thermo Fisher Scientific Co. Ltd). Quantitative test samples were assayed in triplicate and followed up by a TB Green™ Premix Ex Taq™ II KIT to mix 20 ml reaction system that included SYBR Green Premix Ex Taq II (2×) 10 ml, DNA template 2 and 0·8 ml of each primer (10 mM). The parameters of real-time PCR reaction were as follows: degeneration at 95°C for 60 s; PCR reaction at 95°C for 15 s and 60°C for 30 s, forty cycles; dissociation stage.

Calculation and statistical analyses

Feed conversion ratio (FCR) was calculated as daily DMI divided by ADG for each bull. To keep correspondence with the

Table 2. PCR primers for real-time PCR assay

Target species	Primer sequence (5′)	GeneBank accession no.	Size (bp)
Total bacteria	F: CGGCAACGAGCGCAACCC R: CCATTGTAGCACGTGTGTAGCC	AY548787.1	147
Total anaerobic fungi	F: GAGGAAGTAAAAGTCGTAACAAGGTTTC R: CAAATTCACAAAGGGTAGGATGATT	GQ355327.1	120
Total protozoa	F: GCTTTCGWTGGTAGTGTATT R: CTTGCCCTCYAATCGTWCT	HM212038.1	234
Ruminococcus albus	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	CP002403.1	176
Ruminococcus flavefaciens	F: ATTGTCCCAGTTCAGATTGC R: GGCGTCCTCATTGCTGTTAG	AB849343.1	173
Butyrivibrio fibrisolvens	F: ACCGCATAAGCGCACGGA R: CGGGTCCATCTTGTACCGATAAAT	HQ404372.1	65
Fibrobacter succinogenes	F: GTTCGGAATTACTGGGCGTAAA R: CGCCTGCCCCTGAACTATC	AB275512.1	121
Prevotella ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG R: CATCCTATAGCGGTAAACCTTTGG	LT975683.1	74
Ruminobacter amylophilus	F: CTGGGGAGCTGCCTGAATG R: GCATCTGAATGCGACTGGTTG	MH708240.1	102

F, forward; R, reverse.

measurement of BW, feed intake was summarised at a 30-d interval. Data for DMI, BW, ADG and FCR were analysed by the mixed procedure of SAS (Proc Mixed; SAS, 2002)[21] with a 2 (RPFA addition) × 2 (BT addition) completely randomised design, the model was as follows:

$$Y_{ijklm} = \mu + B_i + F_j + G_k + (FG)_{jk} + T_l + (TF)_{jl} + (TG)_{kl} + (TFG)_{jkl} + R_{m:ijk} + \varepsilon_{ijklm}$$

Data for apparent total-tract nutrient digestibility, ruminal fermentation, microbial enzyme activity, microbiota and blood metabolites were analysed by using the model:

$$Y_{ijklm} = \mu + Bi + F_j + G_k + (FG)_{jk} + R_{m:ijk} + \varepsilon_{ijkm}$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, B_i is the random effects of the i^{th} block, F_j is the fixed effects of RPFA addition (j = with or without), G_k is the fixed effects of BT addition (k = with or without), $(FG)_{jk}$ is the RPFA × BT interaction, T_l is the fixed effect of time, $(TF)_{jl}$ is the time × RPFA interaction, $(TG)_{kl}$ is the time × BT interaction, $(TFG)_{jkl}$ is the time × RPFA × BT interaction, R_m is the random effects of the m^{th} bull and ε_{ijklm} is the residual error. For ruminal fermentation, ruminal microbial enzyme activity and microbiota, sampling time was looked as repeated measurements. Means were separated using the PDIFF option in the LSMEANS statement only for interactions that were statistically significant ($P < 0{\cdot}050$). Significant differences were suggested at $P < 0{\cdot}050$.

Results

DM intake, average daily gain and feed conversion ratio

The RPFA × BT interaction was significant ($P < 0{\cdot}05$) for ADG and FCR; ADG increased ($P < 0{\cdot}001$) by 25·2 % and FCR ($P = 0{\cdot}002$) decreased by 16·5 % with the addition of BT in the diet without RPFA but ADG increased ($P = 0{\cdot}026$) by 6·29 % and FCR was unchanged ($P = 0{\cdot}73$) with the addition of BT in the diet with RPFA (Table 3). Addition of RPFA or BT did not affect DMI and BW in bulls.

Apparent total-tract nutrient digestibility and ruminal fermentation

The RPFA × BT interaction was significant ($P < 0{\cdot}05$) for apparent total-tract digestibility of NDF and ADF which increased ($P < 0{\cdot}05$) with supplementation of BT in the diet without RPFA but was unchanged ($P > 0{\cdot}05$) with addition of BT in the diet with RPFA supplementation (Table 4). Apparent total-tract digestibility of DM, OM and CP was elevated ($P < 0{\cdot}05$) due to RPFA or BT addition. The RPFA × BT interaction was significant ($P < 0{\cdot}05$) for ruminal total VFA concentration which increased ($P < 0{\cdot}001$) by 12·6 % when BT was supplemented in the diet without RPFA but was unchanged ($P = 0{\cdot}07$) when BT was added in the diet with RPFA. Ruminal pH was unchanged with RPFA or BT addition. The increase ($P < 0{\cdot}05$) in acetate molar proportion and decrease ($P < 0{\cdot}05$) in propionate molar proportion caused acetate:propionate ratio increase ($P < 0{\cdot}05$) with RPFA or BT addition. Molar proportion of butyrate and valerate was not affected by treatments. Addition of RPFA increased molar proportion of isobutyrate and isovalerate. Addition of BT did not affect isobutyrate molar proportion but increased isovalerate ($P = 0{\cdot}029$) molar proportion. Ruminal ammonia-N concentration was decreased ($P = 0{\cdot}001$) due to RPFA addition but was unchanged with BT addition.

Microbial enzymatic activity and population

The RPFA × BT interaction was not significant for microbial enzymatic activity and population (Table 5). Higher ($P < 0{\cdot}05$) activity of carboxymethyl cellulase, cellobiase, xylanase and protease was observed for bulls receiving RPFA or BT supplementation. Activity of protease and laccase increased ($P < 0{\cdot}05$) but that of α-amylase was unaltered with RPFA addition. Addition of BT did not change the activity of laccase and α-amylase but reduced ($P = 0{\cdot}003$) protease activity. Population of total bacteria, protozoa, *F. succinogenes* and *Rb. amylophilus* was elevated ($P < 0{\cdot}05$), but *B. fibrisolvens* was unchanged with dietary RPFA or BT addition. Population of total fungi, *R. flavefaciens* and *P. ruminicola* increased ($P < 0{\cdot}05$) with RPFA addition but was unchanged with BT addition. In contrast, population of *R. albus* was not affected by RPFA and was elevated ($P = 0{\cdot}033$) by BT addition.

Table 3. Effects of rumen-protected folic acid (RPFA) and betaine (BT) addition on DM intake (DMI), average daily gain (ADG) and feed conversion ratio (FCR) in Angus bulls (*n* 12)*
(Mean values with their standard errors)

	RPFA–		RPFA+			*P*		
Items	BT–	BT+	BT–	BT+	SEM	RPFA	BT	RPFA × BT
DMI (kg/d)	11·6	12·1	11·5	11·6	0·20	0·38	0·40	0·61
Body weight (kg)								
0 d	454	454	454	457	4·7	0·87	0·84	0·92
30 d	493	505	505	512	4·5	0·32	0·31	0·79
60 d	537	559	560	569	4·6	0·09	0·11	0·50
ADG (kg/d)	1·39	1·74	1·75	1·86	0·028	<0·001	<0·001	0·002
FCR† (kg/kg)	8·32	6·95	6·55	6·24	0·166	<0·001	<0·001	0·024

RPFA–, without RPFA; RPFA+, 6 mg/kg DM of folic acid from RPFA during 363–443 d of age; BT–, without BT; BT+, 0·6 g/kg DM of BT during 363–443 d of age; RPFA, RPFA effect; BT, BT effect; RPFA × BT, interaction between RPFA and BT addition.
* *P* values of time for DMI, ADG and FCR were 0·026, 0·001 and 0·449, respectively. The time × RPFA, time × BT and time × RPFA × BT interactions for all the studied variables were not significant ($P > 0{\cdot}05$).
† FCR calculated as daily DMI divided by ADG for each bull.

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Table 4. Effects of rumen-protected folic acid (RPFA) and betaine (BT) addition on total tract nutrient digestibility and ruminal fermentation in Angus bulls (*n* 12)
(Mean values with their standard errors)

	RPFA–*		RPFA+			*P*†		
Items	BT–	BT+	BT–	BT+	SEM	RPFA	BT	RPFA × BT
Digestibility								
DM	0·70	0·74	0·75	0·78	0·006	0·002	0·008	0·30
Organic matter	0·67	0·72	0·73	0·75	0·005	<0·001	0·002	0·12
Crude protein	0·59	0·67	0·65	0·69	0·008	0·016	0·002	0·27
Neutral-detergent fibre	0·59	0·66	0·68	0·69	0·005	<0·001	<0·001	0·007
Acid-detergent fibre	0·47	0·55	0·59	0·59	0·006	<0·001	0·007	0·006
Ruminal fermentation								
pH	6·78	6·66	6·51	6·35	0·082	0·14	0·31	0·63
Total VFA (mM)	78·0	87·8	94·9	98·2	0·80	0·001	0·003	0·049
Mol/100 mol								
Acetate	66·2	67·8	67·3	68·5	0·32	0·025	0·032	0·54
Propionate	23·5	21·6	22·3	20·6	0·22	0·024	0·021	0·55
Butyrate	6·98	7·14	7·16	7·02	0·310	0·90	0·58	0·71
Valerate	0·74	0·81	0·69	0·70	0·037	0·69	0·29	0·63
Isobutyrate	0·98	0·99	1·05	1·22	0·050	0·035	0·14	0·40
Isovalerate	1·59	1·66	1·53	1·98	0·082	0·042	0·029	0·09
A:P	2·82	3·13	3·02	3·33	0·026	0·019	0·008	0·76
Ammonia-N (mg/100 ml)	8·90	8·52	7·28	6·82	0·118	<0·001	0·11	0·88

RPFA–, without RPFA; RPFA+, 6 mg/kg DM of folic acid from RPFA during 363–443 d of age; BT–, without BT; BT+, 0·6 g/kg DM of BT during 363–443 d of age; RPFA, RPFA effect; BT, BT effect; RPFA × BT, interaction between RPFA and BT addition; VFA, volatile fatty acids; A:P, ratio of acetate: propionate.

Table 5. Effects of rumen-protected folic acid (RPFA) and betaine (BT) addition on ruminal microbial enzyme activity and microbiota in Angus bulls (*n* 12)
(Mean values with their standard errors)

	RPFA–		RPFA+			*P*		
Items	BT–	BT+	BT–	BT+	SEM	RPFA	BT	RPFA × BT
Microbial enzyme activity*								
Carboxymethyl cellulase	0·29	0·34	0·33	0·35	0·004	0·023	0·007	0·21
Cellobiase	0·11	0·13	0·13	0·16	0·003	0·010	0·005	0·44
Xylanase	0·80	0·92	0·86	0·96	0·013	0·046	0·015	0·59
Pectinase	0·35	0·45	0·44	0·48	0·011	0·025	0·012	0·23
Laccase	5·32	5·73	6·49	8·29	0·210	0·002	0·30	0·14
α-Amylase	0·14	0·15	0·18	0·19	0·009	0·07	0·68	0·94
Protease	0·63	0·73	0·65	0·89	0·021	0·046	0·003	0·13
Microbiota (copies/ml)								
Total bacteria ($\times 10^{11}$)	2·55	3·20	3·36	3·71	0·051	0·001	0·001	0·17
Total anaerobic fungi ($\times 10^{9}$)	4·68	4·99	6·12	6·46	0·317	0·045	0·62	0·98
Total protozoa ($\times 10^{6}$)	2·49	3·42	3·27	5·39	0·169	0·004	0·002	0·11
Ruminococcus albus ($\times 10^{8}$)	4·03	4·50	4·39	5·90	0·192	0·052	0·033	0·22
Ruminococcus flavefaciens ($\times 10^{9}$)	3·44	3·75	4·93	5·73	0·155	0·001	0·11	0·45
Fibrobacter succinogenes ($\times 10^{10}$)	5·32	5·73	6·49	8·29	0·210	0·002	0·030	0·14
Butyrivibrio fibrisolvens ($\times 10^{9}$)	4·24	4·79	4·67	5·74	0·213	0·14	0·095	0·56
Prevotella ruminicola ($\times 10^{10}$)	2·58	2·26	2·95	3·39	0·083	0·002	0·74	0·052
Ruminobacter amylophilus ($\times 10^{9}$)	1·46	1·66	1·84	2·48	0·076	0·004	0·025	0·18

RPFA–, without RPFA; RPFA+, 6 mg/kg DM of folic acid from RPFA during 363–443 d of age; BT–, without BT; BT+, 0·6 g/kg DM of BT during 363–443 d of age; RPFA, RPFA effect; BT, BT effect; RPFA × BT, interaction between RPFA and BT addition.
* Units of enzyme activity are: carboxymethyl cellulase (μmol glucose/min per ml), cellobiase (μmol glucose/min per ml), xylanase (μmol xylose/min per ml), pectinase (μmol D-galactouronic acid/min per ml), laccase (U/l), α-amylase (μmol glucose/min per ml) and protease (μg hydrolysed protein/min per ml).

Blood metabolites

The RPFA × BT interaction was significant ($P < 0·05$) for blood folate concentration which decreased ($P = 0·002$) by 16·8 % when BT was supplemented in the diet without RPFA but was unchanged ($P = 0·96$) when BT was supplemented in the diet with RPFA (Table 6). Blood concentration of glucose, total protein, albumin, growth hormone and insulin-like growth factor-1 was elevated ($P < 0·05$) by RPFA addition but was unaffected by BT addition. Addition of RPFA or BT decreased blood Hcy concentration but did not affect blood methionine concentration.

No adverse events occurred during the experiment, and no modification to the experimental protocols was made.

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Table 6. Effects of rumen-protected folic acid (RPFA) and betaine (BT) addition on blood metabolites in Angus bulls (*n* 12) (Mean values with their standard errors)

	RPFA–		RPFA+			*P*		
Items	BT–	BT+	BT–	BT+	SEM	RPFA	BT	RPFA × BT
Glucose (mmol/l)	7·49	7·87	8·30	8·93	0·319	0·015	0·09	0·62
Total protein (g/l)	139	142	149	154	1·57	0·046	0·11	0·25
Albumin (g/l)	52·8	54·6	56·0	57·2	1·62	0·048	0·14	0·74
GH (ng/ml)	6·94	8·27	8·78	9·55	0·353	0·043	0·15	0·76
IGF-1 (ng/ml)	216	227	238	254	5·7	0·010	0·11	0·49
Folate (μmol/l)	14·5	12·0	16·7	17·8	0·48	0·002	0·20	0·045
Hcy (μmol/l)	13·1	10·4	11·6	10·1	0·24	0·047	0·001	0·21
Methionine (ng/ml)	12·5	13·7	13·5	14·0	0·35	0·36	0·22	0·62

RPFA–, without RPFA; RPFA+, 6 mg/kg DM of folic acid from RPFA during 363–443 d of age; BT–, without BT; BT+, 0·6 g/kg DM of BT during 363–443 d of age; RPFA, RPFA effect; BT, BT effect; RPFA × BT, interaction between RPFA and BT addition; GH, growth hormone; IGF-1, insulin-like growth factor-1; Hcy, homocysteine.

Discussion

The unchanged DMI with RPFA addition was in accordance with the results of other studies, in which DMI was not affected by FA addition in dairy cows[3] or by RPFA addition in dairy calves[4]. The increase in ADG could be attributed to the greater apparent total-tract nutrient digestibility and ruminal total VFA concentration and might be associated with a positive impact of RPFA addition on protein metabolism. Indeed, dietary RPFA addition increased blood concentration of total protein, albumin, growth hormone and insulin-like growth factor-1 in bulls. The results were consistent with the observed increase in apparent total-tract digestibility of CP. Furthermore, Hcy accepts methyl group from 5-methyl-THF and is converted to methionine to participate in protein synthesis[1]. The decreased Hcy and unchanged methionine in blood indicated that dietary RPFA addition might promote the transfer efficiency of methyl group, thereby facilitating protein synthesis. La *et al.*[4] found that hepatic gene expression responsible for protein synthesis was up-regulated by dietary RPFA addition in dairy calves. The limited response of DMI and the increase in ADG resulted in the decrease in FCR, suggesting that nutrient utilisation efficiency was increased by RPFA supplementation. Similarly, previous studies observed an elevated growth performance and feed utilisation efficiency with RPFA[4] or FA addition[5] in dairy calves. The increase in apparent total-tract digestibility of DM and OM was consistent with the greater ruminal total VFA concentration which suggested that nutrient degradation in the rumen was enhanced by RPFA supplementation. Wang *et al.*[8] observed that ruminal degradability of DM, OM and NDF was elevated with RPFA addition in steers. The positive response of apparent total-tract digestibility of NDF and ADF was in accordance with the higher ruminal acetate molar proportion and was associated with the stimulating effect of RPFA on ruminal microbial growth. The increase in population of total bacteria, fungi, protozoa, *R. albus*, *R. flavefaciens* and *F. succinogenes* caused activity of carboxymethyl cellulase, xylanase, cellobiase, pectinase and laccase increase[22] with RPFA addition. Hence, the rumen fermentation mode was changed to more acetate formation. Ruminal laccase secreted by bacteria and fungi is mainly responsible for the degradation of plant lignin[23]. FA is reduced to THF and then converted to 5,10-methylene-THF and then 5-methyl-THF to transfer one-carbon units, thereby playing a major role in DNA synthesis and cell proliferation[1]. An early *in vitro* study observed that ruminal *R. flavefaciens* growth was retarded when THF or 5-methyl-THF was substituted with FA in the medium[6]. The release ratio of FA from RPFA was 23 % in the rumen. Therefore, the observed increase in ruminal microbial population might be associated with the regulation of dietary RPFA on the one-carbon unit metabolism of microbes. Similarly, previous studies observed that the relative abundance of ruminal cellulolytic bacteria increased with RPFA addition in calves[4] or steers[8]. However, only ruminal fluid was collected for the determination of microbial population and enzyme activity in the current and previous studies[4,8]. There are more bacteria in the solid phase than in the liquid phase of the rumen[22]. Hence, the results observed might be different if the solid-associated bacteria were determined. Ruminal propionate molar proportion decreased but propionate concentration increased by 11·0 % (18·6 and 20·7 mM for RPFA– and RPFA+, respectively) with dietary RPFA addition. The result was consistent with the increase in activity of α-amylase as well as population of *P. ruminicola* and *Rb. amylophilus* and supported the positive response of blood glucose concentration with RPFA addition. Ruminal isobutyrate and isovalerate are derived from the degradation of dietary CP and utilised by micro-organisms to synthesise branched-chain fatty acids and amino acids[24]. The increase in molar proportion of isobutyrate and isovalerate was consistent with the higher activity of protease and population of total protozoa, *P. ruminicola* and *Rb. amylophilus*, indicating that rumen CP degradability increased with RPFA addition, as shown in the study of Wang *et al.*[8]. The increase in CP degradability and protozoa population contributed to an accumulation of ruminal ammonia-N[25,26]. However, the reduced ammonia-N concentration was observed with RPFA addition. Ruminal micro-organisms, especially cellulolytic bacteria, consume ammonia-N to synthesise protein[25]. Fermentable carbohydrates in the rumen provide energy and carbon skeletons for microbial protein synthesis[25]. Considering the positive response of bacteria population and total VFA concentration, the observed decrease in ammonia-N

concentration should be due to an increase in the synthesis of microbial protein. Moreover, other studies reported that ammonia-N assimilation of ruminal *B. fibrisolvens* TC33 increased with FA addition *in vitro*[27] and excretion of urinary purine derivatives was elevated with RPFA addition in steers[8]. The positive impact of RPFA on microbial protein synthesis was also a reason for the increased ADG and apparent total-tract CP digestibility.

Addition of BT at 0·6 g/kg DM did not affect DMI but increased ADG and decreased FCR in bulls consuming a diet with concentrate:forage ratio of 50:50. However, Loest *et al.*[28] reported that DMI, ADG and gain efficiency were unchanged with the addition of 4, 8 and 12 g/d of BT in steers fed a high-concentrate finishing diet, respectively. The level of BT supplemented in the present study (7 g/d of BT) was similar with that in Loest *et al.*[28]. Therefore, the divergent results might be related to the difference in diet composition between the two studies. Loest *et al.*[29] found that ruminal degradation rate of BT was lower in steers fed a high-roughage diet than in steers fed a high-grain diet *in vitro*. The higher forage ratio of the present study compared with Loest *et al.*[28] would cause more supplemented BT escape the degradation in the rumen and reach the small intestine to be absorbed. The increase in apparent total-tract digestibility of DM, OM, CP, NDF and ADF was associated with an enhanced ruminal nutrient degradation, as reflected by the greater ruminal total VFA concentration with BT addition. In addition, post-rumen nutrient digestion might also be stimulated by BT addition. Because of the osmoprotective property of BT, dietary BT addition could improve the structure and function of digestive tract and increase intestinal cell activity, thereby promoting digestive enzyme secretion and nutrient digestion[30]. Similarly, Wang *et al.*[10] observed an increased apparent nutrient digestibility of the total tract with BT addition in dairy cows. The increase in ruminal total VFA concentration and acetate:propionate ratio was due to the positive impact of BT addition on activity of carboxymethyl cellulase, cellobiase, xylanase and pectinase as well as population of total protozoa, *R. albus* and *F. succinogenes*. Ruminal *R. albus* and *F. succinogenes* are dominant fibrolytic bacteria[22], and protozoa is responsible for more than 30 % of fibre degradation in the rumen[31]. Literature demonstrated that BT was an effective osmolyte in bacteria[32] and addition of BT could provide available N and methyl group for ruminal microbial growth[28]. Therefore, the positive response of ruminal microbial population and apparent total-tract nutrient digestibility was observed with BT addition. Similarly, other studies found that dietary BT addition increased fibre digestibility and intestinal gram-positive bacteria abundance in piglets[9] and increased ruminal concentration of total VFA and acetate in dairy cows[10]. The limited response of ruminal propionate concentration (19·7 and 19·6 mM for BT− and BT+, respectively) was in accordance with the unaltered α-amylase activity and explained the lack of response of blood glucose concentration with BT addition. However, the unchanged ammonia-N concentration was not in agreement with the higher protease activity and total protozoa and *Rb. amylophilus* population, indicating that more ammonia-N might be used to synthesise microbial protein. Moreover, the increased rumen total VFA concentration and bacteria population could support an enhanced synthesis of microbial protein with BT addition[25].

The significant RPFA × BT interaction was observed for ADG, ruminal total VFA concentration, apparent total-tract digestibility of NDF and ADF as well as blood folate concentration. The increase in ADG, ruminal total VFA concentration and apparent total-tract digestibility of NDF and ADF and decrease in blood folate concentration indicated that efficiency of one-carbon units transfer and FA utilisation might be increased with BT addition in the diet without RPFA. FA in the form of 5-methyl-THF, 5,10-methylene-THF and 10-formyl-THF provides one-carbon units for the formation of methionine, thymidylate and purine, respectively, thereby playing a major role in the synthesis of protein, DNA and RNA[1,2]. BT provides methyl group for the remethylation of Hcy to methionine and is converted to dimethylglycine[2]. Methyl groups of dimethylglycine are split off via oxidation and transferred to THF to form 5,10-methylene-THF[2,33]. The methylene group of 5,10-methylene-THF could be oxidised to 10-formyl-THF[1,2]. Therefore, addition of BT in the RPFA− diet might increase DNA and protein synthesis and FA utilisation efficiency for both bulls and their ruminal microbes. However, one-carbon metabolism is tightly controlled[33,34]. When the supply of one-carbon units exceeds the need for the synthesis of purine and thymidylate as well as the remethylation of Hcy to methionine, the excess one-carbon units would be removed by cells[33]. Moreover, the pathway of transmethylation (Hcy to methionine) and transsulfuration (Hcy to cystein) could be up-regulated to avoid the excess of methionine[34]. Therefore, limited response was observed for ADG, ruminal total VFA concentration, apparent total-tract NDF and ADF digestibility and blood folate concentration with addition of BT in the RPFA+ diet and for blood methionine concentration with addition of RPFA or/and BT. Similarly, Duplessis *et al.*[35] reported that intramuscular injection of FA did not affect plasma methionine concentration in dairy cows.

Conclusion

Dietary supplementation of RPFA or BT stimulated growth and feed digestion in bulls. The FA and BT were required for ruminal microbial growth, as shown by the increase in cellulolytic bacteria population, enzymatic activity and total VFA concentration with RPFA or BT addition. Both RPFA and BT participate in the one-carbon units cycle and addition of BT in the RPFA− diet increased FA utilisation efficiency. The combined addition of RPFA and BT was not necessary, since the increased magnitude for ADG was greater with addition of BT in the RPFA− diet than in the RPFA+ diet and addition of BT stimulated ruminal VFA production and fibre digestion only when RPFA was not supplemented.

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C. W. and Q. L. designed the experiment. G. W. Z., H. S. D., Z. Z. W., J. Z., C. L. and L. C. conducted the experiment. G. G., W. J. H., S. L. Z. and C. X. P. collected and analysed data. C. W. and Q. L. wrote the manuscript.

The authors declare that there are no conflicts of interest.

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