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Effects of folic acid and riboflavin on growth performance, nutrient digestion and rumen fermentation in Angus bulls

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Abstract

This study examined the influences of coated folic acid (CFA) and coated riboflavin (CRF) on bull performance, nutrients digestion and ruminal fermentation. Forty-eight Angus bulls based on a randomised block and 2 × 2 factorial design were assigned to four treatments. The CFA of 0 or 6 mg of folic acid/kg DM was supplemented in diets with CRF 0 or 60 mg riboflavin (RF)/kg DM. Supplementation of CRF in diets with CFA had greater increase in daily weight gain and feed efficiency than in diets without CFA. Supplementation with CFA or CRF enhanced digestibility of DM, organic matter, crude protein, neutral-detergent fibre and non-fibre carbohydrate. Ruminal pH and ammonia N content decreased and total volatile fatty acids concentration and acetate to propionate ratio elevated for CFA or CRF addition. Supplement of CFA or CRF increased the activities of fibrolytic enzymes and the numbers of total bacteria, protozoa, fungi, dominant fibrolytic bacteria and *Prevotella ruminicola*. The activities of *α*-amylase, protease and pectinase and the numbers of *Butyrivibrio fibrisolvens* and *Ruminobacter amylophilus* were increased by CFA but were unaffected by CRF. Blood concentration of folate elevated and homocysteine decreased for CFA addition. The CRF supplementation elevated blood concentrations of folate and RF. These findings suggested that CFA or CRF inclusion had facilitating effects on performance and ruminal fermentation, and combined addition of CFA and CRF had greater increase in performance than CFA or CRF addition alone in bulls.

Key words: **Folic acid: Riboflavin: Growth performance: Rumen micro-organisms: Bulls**

Folic acid (FA) has the character of accepting and donating one-carbon units and is essential for methionine cycle and purine, pyrimidine and protein synthesis[1,2]. However, the supply in FA based on ruminal synthesis and dietary sources is not sufficient to optimise metabolic efficiency and performance in cattle[2,3]. Approximately 97 % of dietary supplementary FA would be degraded by ruminal microbes[4]. When comparing the supplementation mode at the equal addition level of FA, coated folic acid (CFA) which releases 23 % of FA in the rumen could cause more FA to reach the intestine and be absorbed compared with the supplementation of FA directly. Previous studies observed that addition of CFA elevated daily weight gain and hepatic genes expressions linked to protein synthesis metabolism in Angus bulls[5,6]. Likewise, studies of dairy cows found that FA supply improved energy metabolism efficiency[7], and elevated milk and milk protein yields and blood total sulphur amino acids contents[8]. In the rumen, FA was growth factor of *Ruminococcus albus, R. flavefaciens* and *Butyrivibrio fibrisolvens*[9–11]. Supplementation with CFA in bull diets elevated ruminal volatile fatty acids (VFA) production and fibrolytic micro-organism numbers and stimulated the digestion of DM, organic matter (OM), crude protein (CP) and neutral-detergent fibre (NDF) in the rumen and total tract[4,12]. The findings of studies cited above showed that dietary CFA inclusion was necessary for improving the performance and nutrients digestion in bulls.

Riboflavin (RF), as the precursor substance of FAD and FMN, participates in a variety of redox reactions involved in protein, lipid and carbohydrate metabolisms[13]. The studies *in vitro* found that some strains of *R. albus* growth and cellulose digestion required RF[9,14], and that B group vitamins containing RF addition stimulated protozoa growth[15]. Wu *et al.* observed that RF addition increased total VFA content, total bacteria, fungi and protozoa numbers and nutrients digestibility in the rumen of

Abbreviations: ADG, average daily gain; BW, body weight; CFA, coated folic acid; CP, crude protein; CRF, coated riboflavin; DMI, DM intake; FA, Folic acid; FE, feed efficiency; NDF, neutral-detergent fibre; OM, organic matter; RF, Riboflavin; VFA, volatile fatty acids.

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bulls[16]. However, limited response of production performance was observed in bulls with RF supply[16] and in cows with a B vitamins mixture containing RF addition[17], and this might be due to the fact that 99 % of dietary-supplemented RF was degraded or metabolised in the rumen[4]. Hence, the supplement of coated riboflavin (CRF) should be used in ruminant diets.

In the one-carbon units cycle, the FAD-dependent 5,10-methylene tetrahydrofolate reductase (MTHFR) is in charge of the reductive conversion of 5,10-methylene tetrahydrofolate into 5-methyl-tetrahydrofolate[18]. Methionine synthase (MS) catalyses the reaction that homocysteine accepts the one carbon group of 5-methyl-tetrahydrofolate to convert to methionine and finally loses activity, but methionine synthase reductase (MSR) can restore MS activity via FAD/FMN reductive methylation reaction[18]. Furthermore, Ganji and Kafai reported that dietary RF level was negatively related to serum homocysteine concentration, and the combined supply of FA and RF decreased serum homocysteine concentration in human[19]. Therefore, RF is a key link in FA utilisation[18].

Based on the studies above, supplementing CRF in diets containing CFA might enhance the utilisation efficiency of FA. It was reported that the improvement in performance with B vitamins addition was associated with an enhancement in metabolism efficiency in bulls[5] or cows[7,8]. Hence, it was hypothesised that the increase in average daily gain (ADG) would be greater for combined addition of CFA and CRF than for CFA addition alone in bulls. This study investigated the effects of CFA or/and CRF supply on bull performance, nutrients digestion and ruminal fermentation.

Materials and methods

Animals, experimental design and diets

The animal care followed the guidelines of Animal Care and Use Committee of Shanxi Agriculture University (Taigu, Shanxi, China). This experiment was conducted from August 2020 to October 2020 at a commercial beef farm (Qixian Wanmu Beef Farm, Jinzhong, China). Forty-eight Angus bulls of 247 ± 10·9 d of age and 261 ± 3·31 kg of body weight (BW) were assigned into four treatments according to a 2 × 2 factorial and randomised block (BW) design. The CFA at 0 or 6 mg FA/kg DM was supplemented in diets including CRF 0 or 60 mg RF/kg DM. The additional level of CFA or CRF was determined based on previous studies in bulls[5,12,16]. The CFA and CRF were made based on the procedures described by Wang *et al.*[12]. The CFA product contains 100 g/kg of FA, 550 g/kg of hydrogenated fat (ratio of C16:0-C18:0 = 2:1), 150 g/kg of silicon dioxide, and 200 g/kg of calcium stearate. The CRF product contains 65 g/kg of RF, 460 g/kg of hydrogenated fat (ratio of C16:0-C18:0 = 2:1), 230 g/kg of silicon dioxide, and 240 g/kg of calcium stearate. The ruminal and intestinal FA or RF release percentages were measured by using four bulls with ruminal and duodenal cannulas[6], and were 23 % and 67 % for CFA and 25 % and 69 % for CRF, respectively. The supplementary CFA or CRF was premixed in the bull premix. The diets shown in Table 1 were formulated based on the nutritional requirements of beef cattle in NRC[3] and served twice a day at 06.00 and 18.00 h. The experiment period consisted of 20 d adaptation and 60 d sample collection. During the trial, bulls were kept individually and consumed water and feed *ad libitum*.

Table 1. Ingredient and chemical composition of experimental diets

Item	Contents (g/kg DM)
Ingredients	
Maize silage	500
Maize grain, ground	356
Wheat bran	30
Soyabean meal	43
Cottonseed meal	40
Calcium carbonate	5·0
Salt	5·0
Calcium biphosphate	15
Sodium bicarbonate	5·0
Mineral and vitamin premix*‡	1·0
Chemical composition	
Organic matter	957·4
Crude protein	110·1
Ether extract	41·9
Neutral-detergent fibre	373·8
Acid-detergent fibre	189·3
Non-fibre carbohydrate†	431·6
Ca	6·9
Phosphorus	4·1

CFA, coated folic acid; CRF, coated riboflavin; FA, folic acid; RF, riboflavin.
* Contained per kg premix: 100 mg Co, 8500 mg Cu, 50 000 mg Fe, 30 000 mg Mn, 30 000 mg Zn, 300 mg I, 300 mg Se, 7 500 000 μg vitamin A, 1200, 000 μg vitamin D and 40, 000 μg vitamin E.
† Non-fibre carbohydrate, calculated by 1000 – crude protein – neutral-detergent fibre – fat – ash.
‡ The supplementary CFA or CRF were mixed with the premix before feeding. The CFA at 0 or 6 mg FA/kg DM was supplemented in diets including CRF 0 or 60 mg RF/kg DM. The CFA product contains 100 g/kg of FA, 550 g/kg of hydrogenated fat, 150 g/kg of silicon dioxide and 200 g/kg of calcium stearate. The CRF product contains 65 g/kg of RF, 460 g/kg of hydrogenated fat, 230 g/kg of silicon dioxide and 240 g/kg of calcium stearate.

Sampling and analyses

In the sampling period, the bull BW was individually measured on day 1, 30 and 60. The bull DM intake (DMI) was determined by measuring the amount of feed offered and refusals each day. From day 50 to 56, feed and refusal samples of individual bull were collected daily. Feces of individual bull (250 g) were sampled by the rectum four times a day and with a 6-h interval. These samples were stored at −20°C, mixed by each bull in equal proportion weight (wet basis), dried at 65°C[20], and ground through a 1-mm screen. The feed, orts and feces samples were analysed for DM (method 934·01), OM (method 942·05), nitrogen (method 976·05) and acid-detergent fibre (ADF; method 973·18) by using the method of AOAC[20], and NDF by the procedure of Van Soest *et al.*[21]. Acid-insoluble ash content as an indicator for digestibility was measured according to procedure described by Van-Keulen and Young[22].

On day 57 and 58, samples of ruminal fluid for each animal was taken with a stomach tube daily at 05.00, 08.00, 11.00 and 14.00 h. To avoid saliva contamination, the first collected sample of 200 ml was abandoned and the subsequent 200 ml was kept, determined for pH, and filtered using four layers of cotton gauze. Filtrates (10 ml) used for the measurement of ammonia-N and VFA concentrations were stored at −20°C, and that (30 ml) for the determination of microbial population and enzyme activity were stored at −80°C[12].

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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
R. albus	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	CP002403·1	176
R. flavefaciens	F: ATTGTCCCAGTTCAGATTGC R: GGCGTCCTCATTGCTGTTAG	AB849343·1	173
B. fibrisolvens	F: ACCGCATAAGCGCACGGA R: CGGGTCCATCTTGTACCGATAAAT	HQ404372·1	65
F.succinogenes	F: GTTCGGAATTACTGGGCGTAAA R: CGCCTGCCCCTGAACTATC	AB275512·1	121
Rb. amylophilus	F: CTGGGGAGCTGCCTGAATG R: GCATCTGAATGCGACTGGTTG	MH708240·1	102
P. ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG R: CATCCTATAGCGGTAAACCTTTGG	LT975683·1	74

Ruminal VFA concentration was analysed using a GC (GC9860; Jinan Qida Analytical Instrument Co., Ltd) and the internal standard was 2-ethylbutyric acid. Ruminal ammonia-N content was measured using a colorimetric spectrophotometer (UV-2450, Jinan Qida Analytical Instrument Co., Ltd)[20]. Ruminal fluid samples were sonicated in a ice-water bath with 20-s pulse rate and 10 min and separated the supernatant by centrifuging at 30 000 ***g*** and 4°C for 10 min to measure enzyme activity according to the method of Agarwal *et al.*[23] and Rodrigues *et al.*[24]. The 1·2 ml of homogenised ruminal fluid was applied to the extraction of micro-organism DNA based on the procedures (RBB + C) described by Yu and Morrison[25]. The extracted DNA was evaluated quality and quantity by using the agarose gel electrophoresis and NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Micro-organism DNA primer sequences were designed according to the reports of Zhou *et al.*[26] and Stevenson and Weimer[27] and presented in Table 2. The standard of each micro-organism DNA was produced using regular PCR, purified with the PureLinkTM Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific Co., Ltd) and then quantified using a spectrophotometer. The copy number of micro-organism DNA standard was evaluated based on length and mass of each PCR product by using a serial 10-fold diluent from 10^1 to 10^8 DNA copies[28]. The amplification and detection of real-time PCR were carried out in a Chromo 4™ system (Bio-Rad). The samples of real-time PCR amplification were analysed in triplicate by using the Biotools QuantiMix EASY SYG Kit (B&M Labs, S. A.). The PCR assay condition was initial denaturation (1 cycle of 50°C for 2 min and 95°C for 2 min) and primer annealing and product elongation (40 cycles of 95°C for 15 s and 60°C for 1 min).

On day 59, blood samples of each bull were taken via coccygeal vein using a Vacutainer system (10 ml, Jinan Qiansi Biotechnology Co. Ltd) at 09.00 h, centrifuged at 2000 ***g*** and 4°C for 15 min to separate serum and kept at −80°C. Concentrations of glucose, albumin, total protein, urea nitrogen, folate, homocysteine and RF in serum were analysed by a PT-3502PC Microplate reader (Beijing Putian Xinqiao Technology Co., Ltd) and corresponding commercial kits designed for bovine (Meilian Biotechnology Co., Ltd).

Calculation and statistical analyses

For each bull, feed efficiency (FE) was calculated as ADG divided by DMI. Supplementary CFA at 0 or 6 mg of FA/kg and CRF at 0 or 60 mg RF/kg were used in a randomised block and 2 × 2 factorial design. The MIXED procedure of SAS[29] was used to analyse the variables of current study. The data of DMI were averaged by every 30 d, and DMI, BW, ADG and FE were analysed by using the model, $Yijklm = \mu + Bi + G_j + H_k + (GH)_{jk} + T_l + (TG)_{jl} + (TH)_{kl} + (TGH)_{jkl} + R_{m:ijk} + \epsilon_{ijklm}$. Rumen fluid samples were analysed using the average value of all sampling time, and nutrients digestibility and ruminal fermentation and blood parameters were analysed by using the model, $Yijklm = \mu + Bi + G_j + H_k + (GH)_{jk} + R_{m:ijk} + \epsilon_{ijkm}$. In the model, $Yijklm$ is the dependent variable, μ is the overall mean, B_i is the random effects of the i^{th} block, G_j is the fixed effects of CFA supplementation (j = 0 or 6 mg/kg), H_k is the fixed effects of CRF supplementation (k = 0 or 60 mg/kg), $(GH)_{jk}$ is the CFA × CRF interaction, T_l is the fixed effect of time; $(TG)_{jl}$ is the time × CFA interaction, $(TH)_{kl}$ is the time × CRF interaction, $(TGH)_{jkl}$ is the time × CFA × CRF interaction, R_m is the random effects of the m^{th} bull, and ϵ_{ijklm} is the the residual error. Mean separations of difference tests was done by using the PDIFF option in the LSMEANS statement (Tukey's test) in SAS[29]only for influences that were significant at $P < 0{\cdot}05$. The significant effects for the factors were suggested at $P < 0{\cdot}05$.

Results

Body weight change, DM intake and feed efficiency

The significant CFA × CRF interaction was noted on ADG and FE; the increases in ADG and FE were higher ($P < 0{\cdot}05$) for providing CRF in the CFA+ diets compared with the CFA diets (Table 3). The DMI and 0- and 30-d BW among treatments

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Table 3. Effects of coated folic acid (CFA) and coated riboflavin (CRF) on DM intake, average daily gain and feed efficiency in dairy bulls (Mean values with their standard errors of the mean)

	CFA−*		CFA+			*P*†		
Item	CRF−	CRF+	CRF−	CRF+	SEM	CFA	CRF	CFA × CRF
DM intake (kg/d)	8·26	8·42	8·44	8·18	0·058	0·78	0·65	0·071
Body weight (kg)								
0 d	261	263	264	263	3·31	0·87	0·89	0·86
30 d	302	310	307	315	3·22	0·43	0·25	0·94
60 d	349	352	355	372	3·27	0·031	0·081	0·76
Average daily gain (kg/d)	1·45	1·63	1·53	1·81	0·036	0·007	0·009	0·017
Feed efficiency (kg/kg)‡	0·17	0·19	0·18	0·22	0·002	0·012	0·008	0·040

FA, folic acid; RF, riboflavin; FE, feed efficiency; ADG, average daily gain.
* CFA-, CFA, CFA+, 6 mg FA/kg DM as CFA, CFR-, without CFR, CFR+, 60 mg RF/kg DM as CRF.
† CFA, CFA effect; CRF, CRF effect; CFA × CRF, the interaction between CFA and CRF addition. The *P*-value of time for DM intake, average daily gain and feed efficiency were 0·001, 0·001 and 0·314, respectively. The time × CFA, time × CRF and time × CFA × CRF interaction for all the studied variables were not significant ($P > 0·05$).
‡ FE was calculated as ADG divided by DMI.

had no significant difference. The 60-d BW elevated ($P < 0·05$) with CFA addition and was unaltered with CRF supplementation.

Nutrients digestibility and ruminal fermentation

Significant CFA × CRF interaction was not observed on total tract nutrients digestibility and ruminal fermentation parameters (Table 4). The digestibility of DM, OM, CP, NDF and NFC in the total tract enhanced ($P < 0·05$) for CFA or CRF addition. The digestibility of ADF in the total tract elevated ($P = 0·019$) for CFA supply but was unaltered for CRF supply. Ruminal pH reduced ($P < 0·05$), but total VFA content elevated ($P < 0·05$) for CFA or CRF supplementation. Addition of CFA elevated ($P < 0·05$) acetate percentage and acetate to propionate ratio but decreased ($P = 0·003$) the percentage of propionate. Supplementation with CRF did not influence the percentages of acetate and propionate but elevated ($P = 0·011$) acetate to propionate ratio. The percentages of butyrate, valerate, isobutyrate and isovalerate were not influenced by CFA or CRF supplementation. Ammonia-N content reduced ($P < 0·05$) for CFA or CRF supply.

Rumen enzyme activity and micro-organism number

The CFA × CRF interaction was not significant for rumen enzyme activity and microbial number (Table 5). The CFA or CRF supply enhanced ($P < 0·05$) the activities of carboxymethyl cellulase, cellobiase and xylanase. The activities of pectinase, α-amylase and protease were enhanced ($P < 0·05$) by CFA but were unaffected with CRF supply. Supplementation of CFA or CRF elevated ($P < 0·05$) numbers of total bacteria, fungi, protozoa, *R. albus*, *R. flavefaciens*, *Fibrobacter succinogenes* and *Prevotella ruminicola*. The numbers of *B. fibrisolvens* and *Ruminobacter amylophilus* elevated ($P < 0·05$) for CFA supply but did not change with CRF addition.

Blood parameters

Significant CFA × CRF interaction was not found for blood parameters (Table 6). Blood glucose concentration was elevated ($P = 0·023$) by CFA but was unaltered with CRF addition. Dietary CFA or CRF supply elevated ($P < 0·05$) blood contents of total protein and albumin but did not influence urea nitrogen. Blood content of folate increased ($P = 0·007$), homocysteine reduced ($P = 0·028$) and RF was unchanged for bulls with CFA supplementation. Blood concentrations of folate and RF increased ($P < 0·05$) and homocysteine was unaltered for bulls with CRF inclusion.

Discussion

The CFA contains 100 g/kg of FA and CRF contains 65 g/kg of RF. The ruminal and intestinal disappearance rates were 23 % and 67 % for CFA, and 25 % and 69 % for CRF, respectively. The addition levels of CFA and CRF were 6 mg FA/kg DM and 60 mg RF/kg DM, respectively. Based on daily DMI, addition of CRF provided 126 and 123 mg of RF in the rumen and 349 and 339 mg of RF in the intestine for bulls in the CRF+ and CFA + CRF+ groups, and addition of CFA provided 11·6 and 11·3 mg of FA in the rumen and 33·9 and 32·9 mg of FA in the intestine for bulls in the CFA+ and CFA + CRF+ groups. Dietary added fat due to the coating of CFA and CRF was 0·42, 0·033 and 0·45 g/kg DM for CRF+, CFA+ and CFA + CRF+, respectively, and had limited impacts on the total fat percentage of the diets.

As in previous study of Liu *et al.*[5], CFA supplement increased ADG and FE without affecting DMI of bulls. Likewise, Levesque *et al.*[30] reported that dietary addition of FA improved growth performance but had no effect on feed intake in calves. The changes of ADG and FE were in line with the elevation in nutrients apparent digestibility and rumen total VFA content and were likely also due to the fact that CFA supply improved metabolism efficiency of bulls. The increase in blood contents of total protein and albumin combined with the unchanged urea nitrogen content showed that dietary nitrogen utilisation efficiency might be improved with supplementation of CFA in bull diets. The increment in blood folate and reduction in blood homocysteine suggested that supplementary FA was absorbed effectively and promoted the remethylation of homocysteine to methionine, facilitating an increase in ADG. FA participates in protein synthesis metabolism by transmitting one-carbon units, and homocysteine receives the methyl group of 5-methyl-tetrahydrofolate to generate methionine[1]. Studies indicated that FA addition increased hepatic gene expression linked

Table 4. Effects of coated folic acid (CFA) and coated riboflavin (CRF) on nutrient apparent digestibility and ruminal fermentation in Angus bulls (Mean values with their standard errors of the mean)

	CFA−*		CFA+			*P*†		
Item	CRF−	CRF+	CRF−	CRF+	SEM	CFA	CRF	CFA × CRF
Digestibility (%)								
DM	66·3	70·0	68·2	69·5	0·19	0·023	0·015	0·77
Organic matter	68·7	73·3	72·7	74·8	0·20	0·016	0·032	0·081
Crude protein	72·6	74·7	75·2	77·7	0·36	0·022	0·031	0·13
Neutral-detergent fibre	52·0	55·9	55·4	57·0	0·66	0·021	0·009	0·57
Acid-detergent fibre	39·5	41·1	42·7	44·0	0·88	0·019	0·095	0·18
Non-fibre carbohydrate	82·2	87·0	86·2	88·9	0·69	0·043	0·012	0·15
Ruminal fermentation								
pH	6·74	6·29	6·36	6·21	0·031	0·013	0·012	0·26
Total VFA (mM)‡	115	130	127	134	1·97	0·032	0·025	0·47
Mol/100 mol								
Acetate (A)	68·6	69·8	70·9	71·3	0·46	0·037	0·14	0·34
Propionate (P)	18·2	18·2	16·7	16·2	0·54	0·003	0·28	0·11
Butyrate	10·5	9·32	9·68	9·82	0·29	0·59	0·12	0·45
Valerate	0·94	0·85	0·93	1·01	0·029	0·21	0·91	0·34
Isobutyrate	0·70	0·72	0·69	0·71	0·020	0·82	0·60	0·26
Isovalerate	1·04	1·09	1·13	0·99	0·050	0·96	0·59	0·17
A:P	3·76	3·84	4·25	4·40	0·016	0·009	0·011	0·057
Ammonia N (mg/100 ml)	6·45	5·66	5·89	5·28	0·074	0·006	0·015	0·74

FA, folic acid; RF, riboflavin; VFA, volatile fatty acids.
* CFA-, without CFA; CFA+, 6 mg FA/kg DM as CFA; CFR-, without CFR; CFR+, 60 mg RF/kg DM as CRF.
† CFA, CFA effect; CRF, CRF effect; CFA × CRF, the interaction between CFA and CRF addition.
‡ VFA.

Table 5. Effects of coated folic acid (CFA) and coated riboflavin (CRF) on ruminal enzyme activity and microflora in Angus bulls (Mean values with their standard errors of the mean)

	CFA−*		CFA+			*P*†		
Item	CRF−	CRF+	CRF−	CRF+	SEM	CFA	CRF	CFA × CRF
Microbial enzyme activity‡								
Carboxymethyl cellulase	0·29	0·33	0·38	0·40	0·006	0·028	0·037	0·56
Cellobiase	0·15	0·16	0·18	0·21	0·002	0·032	0·021	0·13
Xylanase	0·86	0·97	0·98	1·06	0·019	0·010	0·022	0·72
Pectinase	0·56	0·58	0·60	0·62	0·007	0·023	0·17	0·94
α-amylase	0·59	0·62	0·64	0·67	0·018	0·011	0·084	0·84
Protease	0·58	0·60	0·64	0·68	0·012	0·044	0·47	0·63
Ruminal microflora (copies/ml)								
Total bacteria, $\times 10^{11}$	1·12	1·29	1·27	1·32	0·19	0·036	0·010	0·15
Total anaerobic fungi, $\times 10^{9}$	1·94	2·83	2·53	3·52	0·26	0·024	0·037	0·72
Total protozoa, $\times 10^{6}$	2·29	3·61	4·51	5·29	0·10	0·019	0·023	0·18
R. albus, $\times 10^{8}$	1·40	2·18	2·11	2·61	0·15	0·018	0·034	0·21
R. flavefaciens, $\times 10^{9}$	2·45	3·56	3·48	4·72	0·084	0·022	0·008	0·69
F. succinogenes, $\times 10^{10}$	4·81	6·48	5·51	7·20	0·063	0·034	0·016	0·92
B. fibrisolvens, $\times 10^{10}$	1·54	1·78	2·18	2·27	0·085	0·029	0·14	0·33
P. ruminicola, $\times 10^{10}$	6·47	6·94	7·46	8·35	0·061	0·015	0·032	0·091
Rb. amylophilus, $\times 10^{8}$	6·97	7·23	7·84	8·45	0·25	0·027	0·17	0·16

FA, folic acid; RF, riboflavin.
* CFA- , without CFA; CFA+, 6 mg FA/kg DM as CFA; CFR-, without CFR; CFR+, 60 mg RF/kg DM as CRF.
† CFA, CFA effect; CRF, CRF effect; CFA × CRF, the interaction between CFA and CRF addition.
‡ Units of enzyme activity are carboxymethyl cellulase (μmol glucose/min/ml), cellobiase (μmol glucose/min/ml), xylanase (μmol xylose/min/ml), pectinase (μmol D-galactouronic acid/min/ml), α-amylase (μmol glucose/min/ml) and protease (μg hydrolysed protein/min/ml).

to protein synthesis metabolism in bulls[5], promoted murine myoblasts differentiation by activating the Akt pathway[31] and improved energy metabolism efficiency in dairy cows receiving vitamin B_{12} addition[7]. The results of Petitclerc *et al.*[32] that intramuscular injection of FA increased weight gain of heifers during a 5-week period following weaning also indicated that metabolism efficiency might be improved by FA addition. The elevation in digestibility of DM and OM in the total tract suggested that nutrients digestibility in the rumen and post-rumen were probably enhanced by CFA supply. FA is a prime requisite for cell growth and multiplication[1]. Studies verified that FA addition was required for ruminal cellulose digestion *in vitro*[9] and improved the structure and function of small intestine in monogastric animals[33,34]. In addition, it had been reported that digestibility of DM, CP and NDF in the rumen and total tract enhanced with CFA supplementation in steers[12], and DM digestibility in

Table 6. Effects of coated folic acid (CFA) and coated riboflavin (CRF) on blood parameters in Angus bulls (Mean values with their standard errors of the mean)

	CFA−*1		CFA+			*P*†		
Item	CRF−	CRF+	CRF−	CRF+	SEM	CFA	CRF	CFA × CRF
Glucose (μmol/l)	352	360	416	423	9·35	0·023	0·84	0·44
Total protein (μg/ml)	789	887	882	921	11·6	0·012	0·025	0·076
Albumin (μg/ml)	329	358	354	371	3·75	0·019	0·005	0·48
Urea nitrogen (μmol/l)	195	187	201	203	8·96	0·089	0·18	0·36
Folate (μmol/l)	13·4	14·5	16·0	16·9	0·16	0·007	0·008	0·76
Homocysteine (μmol/l)	11·1	9·20	7·18	8·48	0·33	0·028	0·87	0·52
Riboflavin (μg/l)	2·57	2·93	2·69	3·00	0·042	0·16	0·005	0·43

FA, folic acid; RF, riboflavin.
* CFA- , without CFA; CFA+, 6 mg FA/kg DM as CFA; CFR-, without CFR, CFR+, 60 mg RF/kg DM as CRF.
† CFA, CFA effect; CRF, CRF effect; CFA × CRF, the interaction between CFA and CRF addition.

post-rumen and total tract elevated for FA supply *in vitro*[35]. Similar with the observation of Wang *et al.*[12], CFA supply reduced ruminal pH but elevated total VFA content in bulls. Nevertheless, the mean ruminal pH for bulls receiving CFA supply was 6·30 and was over the critical pH value of 6·0 for cellulolytic bacteria growth and cellulosic materials degradation[36]. The higher ruminal total VFA content and acetate proportion corresponded with the increase in total tract digestibility of NDF and ADF and were probably attributed to the elevation in fibrolytic enzyme (carboxymethyl cellulase, cellobiase, xylanase and pectinase) activity and total bacteria, protozoa and fungi numbers. Ruminal fibrolytic enzymes were produced by cellulolytic species (*R. albus*, *R. flavefaciens*, *F. succinogenes* and *B. fibrisolvens*), protozoa and fungi, and hydrolysed cellulosic materials to acetate[37]. It was reported that ruminal protozoa were responsible for approximately 34 % of total microbial degradation of fibre, and fungi were capable of releasing lignin from plant particles[38,39]. The present results reflected a stimulatory influence of FA on ruminal micro-organism growth and nutrients digestion. FA functions in the cycle of one-carbon units of micro-organisms[1,10]. Studies verified that FA was necessary for some species of *Ruminococcus* and *B. fibrisolvens* *in vitro*[9–11], and that FA supplementation increased acetate content and *Lactobacillus* relative abundance in the caecum of piglets[40] as well as folate synthesis and activity of the bacteria *in vitro*[41]. The change of acetate percentage resulted in an increase in acetate to propionate ratio, causing the fermentation mode shift to more acetate yielding, reflected as the decrease in propionate percentage. Likewise, previous studies observed that CFA supply increased ruminal total VFA content, acetate proportion, microbial counts and enzymes activities in steers[12] or Angus bulls[5,6]. The reduction in ruminal ammonia-N content was probably attributed to the increase in total numbers of bacteria. Rumen ammonia-N is the primary source of cellular nitrogen of bacterial species and is a predictor of conversion efficiency of feed nitrogen to microbial nitrogen[42]. Moreover, studies reported that FA addition *in vitro* increased the utilisation efficiency of ammonia-N by bacteria[11], and CFA supply in steer diets increased the excretion of urinary total purine derivatives, an indicator of microbial protein amount[12,43]. Likewise, it was reported that ammonia-N content reduced with FA addition *in vitro*[35] or in steers[12]. The increase in activities of α-amylase and protease were in line with the increment in numbers of *Rb. amylophilus*, *P. ruminicola* and *B. fibrisolvens* and were a reason for the elevation of NFC and CP digestibility. Furthermore, the elevation in NFC apparent digestibility was likely the reason of the increment in blood glucose concentration, since ruminal propionate concentration (22·3 and 21·5 mM for CFA- and CFA+, respectively) was unaltered with CFA addition. Blood glucose of approximately 80 % originates from propionate gluconeogenesis, and another 20 % is absorbed from the small intestine glucose, products of NFC digestion[44]. In addition, studies in dairy cows found that FA addition upregulated genes expression related to gluconeogenesis metabolism[45] and promoted utilisation efficiency of glucose precursors for gluconeogenesis when vitamin B_{12} was sufficient in diets[46]. Similarly, Duplessis *et al.*[47] observed that plasma glucose concentration increased for cows receiving FA supplement.

The absence of response in DMI indicated that the increase in ADG and FE with addition of CRF should be attributed to an improvement in utilisation efficiency of nutrients or/and energy. The increment in total tract nutrients digestibility and rumen total VFA content reflected that CRF supply stimulated nutrients utilisation in bulls. The higher blood RF content showed that CRF supply improved RF status of bulls. In the form of FMN or FAD, RF assists in the generation of ATP by transferring electrons[13], and studies indicated that RF supply improved energy-yielding metabolism in human[48] or mice[49]. Similarly, Wu *et al.*[16] observed that DMI was unaltered, but ADG tended to increase linearly when RF supplementation was increased from 30, 60 to 90 mg/kg DM in Holstein bulls. Majee *et al.*[17] observed an unchanged feed intake and tended increased milk yield with inclusion of a B–vitamins mixture containing RF in cow diets. The apparent digestibility of DM and OM elevated for CRF addition. The elevation in content of rumen total VFA suggested that digestion of nutrients in the rumen was stimulated by CRF supply. Moreover, studies had verified that RF participated in cellular division and differentiation and was essential for maintaining normal structure and function of small intestine[50]. Likewise, Wu *et al.*[16] found increased ruminal and total tract digestibility of DM and OM with RF supply in Holstein bulls. The increment in CP apparent digestibility was in line with the elevation in blood contents of total protein and albumin, supporting the positive response of growth

performance for bulls with CRF supply. The reduction in rumen pH (from 6·55 to 6·25) for CRF supply was consistent with the changes of total VFA content and had no negative influence on feed degradation and microbial growth[36]. Ruminal acetate was the principal product formed by cellulolytic bacteria, protozoa and fungi[37]. Hence, the elevation in total VFA content and acetate to propionate ratio was related to the increase in activities of carboxymethyl cellulase, cellobiase and xylanase as well as numbers of bacteria, fungi, protozoa and fibrolytic bacteria (*R. albus, R. flavefaciens* and *F. succinogenes*), suggesting that the mode of ruminal fermentation was shifted to yield more acetate due to CRF supplementation. In the rumen, FAD accepts hydrogen ions produced in carbohydrate fermentation and transfers them to NAD to generate NADH, which participates in the reduction reaction in microbes[51]. Likewise, the *in vitro* studies found that RF supply was required for some strains of *R. albus* growth[9] and cellulose digestion[14], and B group vitamins containing RF addition stimulated the growth of protozoa[15]. The limited response in propionate proportion was associated with the unchanged numbers of *B. fibrisolvens* and *Rb. amylophilus* and activity of *α*-amylase and was in line with the unchanged blood glucose content with CRF supplementation. The current results were similar with Wu *et al.*[16], reflecting that addition of CRF mainly favoured the growth of microbes involved in the fermentation of dietary fibre. Moreover, Beaudet *et al.*[52] proposed that starch-degrading bacteria produced more RF compared with fibre degrading microbes. The reduction in ammonia-N content was probably related to an enhancement in the synthesis of micro-organism protein. Wu *et al.*[16] noted that dietary RF supply elevated urinary total purine derivatives excretion in bulls.

The significant CFA × CRF interaction was noted on ADG and FE, showing that the increased magnitudes of ADG and FE were higher when providing CRF in diets with CFA compared with diets without CFA. The CFA × CRF interaction was not significant on rumen total VFA content and total tract nutrients digestibility. Nevertheless, when the CFA was supplemented in diets, blood concentration of folate was higher but that of homocysteine was no difference for bulls with CRF addition compared with those without CRF addition, indicating that FA utilisation efficiency might be elevated by CRF addition. The RF is a key link in FA utilisation, and FAD and FMN are coenzymes of MTHFR and MSR[18]. The MTHFR is responsible for the conversion of 5,10-methylene-tetrahydrofolate into 5-methyl-tetrahydrofolate, and MSR activates MS which catalyses homocysteine to accept the methyl group of 5-methyl-tetrahydrofolate to convert to methionine[18]. Therefore, the responses of ADG and FE to CFA × CRF interaction were likely due to an increase in FA utilisation efficiency when CRF was supplemented in the CFA diets.

Conclusion

Supplementation with CFA of 6 mg FA/kg DM or CRF of 60 mg RF/kg DM promoted bull growth, and this was associated with the elevation in nutrients digestibility and ruminal VFA production. Addition of CFA or CRF stimulated growth of ruminal micro-organisms responsible for fibre degradation and changed rumen fermentation pattern to produce more acetate. The increase in ADG was greater for combined addition of CFA and CRF than for CFA or CRF addition alone, and CRF supply increased blood folate concentration in bull. Therefore, the utilisation efficiency of CFA might be enhanced with CRF addition in bull diets.

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

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