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Effects of folic acid and cobalt sulphate supplementation on growth performance, nutrient digestion, rumen fermentation and blood metabolites in Holstein calves

Yongjia Liu, Jing Zhang, Cong Wang, Qiang Liu*, Gang Guo, Wenjie Huo, Lei Chen, Yanli Zhang, Caixia Pei and Shuanlin Zhang

College of Animal Science, Shanxi Agricultural University, Taigu 030801, Shanxi Province, People's Republic of China

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Abstract

To investigate the influences of cobalt (Co) and folic acid (FA) on growth performance and rumen fermentation, Holstein male calves (*n* 40) were randomly assigned to four groups according to their body weights. Cobalt sulphate at 0 or 0·11 mg Co/kg DM and FA at 0 or 7·2 mg/kg DM were used in a 2 × 2 factorial design. Average daily gain was elevated with FA or Co supplementation, but the elevation was greater for supplementing Co in diets without FA than with FA. Supplementing FA or Co increased DM intake and total-tract nutrient digestibility. Rumen pH was unaltered with FA but reduced with Co supplementation. Concentration of rumen total volatile fatty acids was elevated with FA or Co inclusion. Acetate percentage and acetate to propionate ratio were elevated with FA inclusion. Supplementing Co decreased acetate percentage and increased propionate percentage. Activities of xylanase and *α*-amylase and populations of total bacteria, fungi, protozoa, *Ruminococcus albus*, *Fibrobacter succinogenes* and *Prevotella ruminicola* increased with FA or Co inclusion. Activities of carboxymethyl-cellulase and pectinase increased with FA inclusion and population of methanogens decreased with Co addition. Blood folates increased and homocysteine decreased with FA inclusion. Blood glucose and vitamin B_{12} increased with Co addition. The data suggested that supplementing 0·11 mg Co/kg DM in diets containing 0·09 mg Co/kg DM increased growth performance and nutrient digestibility but had no improvement on the effects of FA addition in calves.

Key words: **Cobalt sulphate: Folic acid: Growth performance: Rumen fermentation: Calves**

Rumen microbes require cobalt (Co) for the synthesis of vitamin B_{12}, a cofactor of methylmalonyl-CoA mutase and methionine synthase, which is essential in the metabolisms of carbohydrate, protein and lipid[1,2]. The level of Co in diets was positively related to rumen vitamin B_{12} synthesis in steers[3,4]. The recommended Co requirement by the National Research Council was 0·11 mg/kg DM for dairy cattle[1]. However, some studies reported that 0·2 mg Co/kg DM was required for growing cattle to support growth, folate metabolism and blood vitamin B_{12} concentration[5,6]. Studies *in vivo* reported that dietary Co inclusion increased DM intake (DMI), average daily gain (ADG) and rumen propionate production in steers[3,4] and total-tract nutrient digestibility in lambs[7]. Studies *in vitro* demonstrated that vitamin B_{12} was required for the growth and propionate production of rumen *Prevotella ruminicola*[8,9]. However, information about the influences of dietary Co supplementation on nutrient digestibility and rumen microflora was limited in dairy calves.

Folic acid (FA) functions in DNA synthesis and protein metabolism and is necessary for rumen microbes and animals[1,10]. Early studies of *in vitro* reported that FA addition increased cellulose digestion[11], and that tetrahydrofolate (THF) or 5-methyl-THF was needed for *Ruminococcus flavefaciens* growth[12]. Furthermore, studies have also proven the inclusion of FA increased ADG, total-tract nutrient digestibility, rumen total volatile fatty acids (VFA) content and microbiota abundance in calves[13,14]. Parnian-Khajehdizaj *et al.*[15] reported increased post-ruminal and total-tract DM digestibility with FA inclusion *in vitro*. These observed positive outcomes with FA dietary inclusion influence one carbon metabolism[10,16]. Vitamin B_{12} dependent methionine synthase is required in the process that 5-methyl-THF donors a methyl group to homocysteine (Hcy) to regenerate methionine[10]. Preynat *et al.*[16] reported that milk production of dairy cows was unchanged with intramuscular injection of FA but tended to increase for FA and vitamin B_{12} injection. Graulet *et al.*[17] reported that when vitamin supplements were

Abbreviations: ADF, acid-detergent fibre; ADG, average daily gain; BW, body weight; Co, cobalt; DMI, DM intake; FA, folic acid; Hcy, homocysteine; NDF, neutral-detergent fibre; OM, organic matter; THF, tetrahydrofolate; VFA, volatile fatty acid.

* **Corresponding author:** Qiang Liu, email liuqiangabc@163.com

top-dressed with the cow morning meal, plasma glucose concentration was higher and hepatic lipids content was lower for FA and vitamin B_{12} addition than for FA addition. The data suggested that when FA was supplemented together with vitamin B_{12}, utilisation efficiency of the two vitamins might be improved.

Given the roles of FA and vitamin B_{12} in methionine cycle as well as the results of studies above, it was hypothesised that calves with combined supplementation of FA and Co might have greater ADG than those receiving FA or Co supplementation alone. Therefore, the present study was undertaken in an attempt to elucidate the influences of dietary inclusion of FA or/and cobalt sulphate on growth performance and rumen fermentation in Holstein calves.

Materials and methods

Holstein calves, treatments and diets

The protocol was approved by the Animal Care and Use Committee of Shanxi Agriculture University. Forty Holstein male calves (88·1 (SEM 13·3) kg of body weight (BW) and 63 (SEM 9·2) d of age) were blocked by BW and randomly divided into four treatments. Cobalt sulphate at 0 (Co−) or 0·11 mg Co/kg DM (Co+) and FA at 0 (FA−) or 7·2 mg/kg DM (FA+) were used in a 2 × 2 factorial design. Supplemented FA (980 g FA/kg) and cobalt sulphate ($CoSO_4 \cdot 7H_2O$, 210 g Co/kg) were purchased commercially, mixed into the mineral and vitamin premix and then into the concentrate before the trial. The supplementation dose of FA was ascertained based on the results of Wang *et al.*[13], and Co was determined on the results of Stangl *et al.*[5] and Schwarz *et al.*[6], where dietary 0·20 mg Co/kg DM could support the maximum growth and normal folate metabolism in growing bulls. Basal diets of calves (Table 1) were formulated according to the recommendations of National Research Council[1] and contained 0·09 mg Co/kg DM and 0·31 mg FA/kg DM. Calves were housed individually in a pen of 2·5 m × 3 m, fed at 07.30 and 19.30 hours daily and had free access to diets and drinking water. The experiment included 20 d of adaptation period and 60 d of data collection period.

Table 1. Ingredient and chemical composition of the basal diet used

Ingredients	Contents (g/kg DM)
Maize silage	500
Maize grain, ground	259
Wheat bran	30
Soyabean meal	140
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	935
Crude protein	129
Neutral-detergent fibre†	331
Acid-detergent fibre	194
Ca	6·5
P	4·6
Cobalt (mg/kg)	0·09

* Contained per kg premix: 1600 mg Cu, 8000 mg Mn, 7500 mg Zn, 120 mg I, 1600 mg vitamin A, 600 mg vitamin D and 5000 mg vitamin E.
† Non-fibre carbohydrate calculated by 1000-CP-NDF-Fat-Ash.

Sampling and analyses

During the data collection period, individual animal was weighed before the morning feeding on days 0, 30 and 60. The feed offered was weighted at 07.30 and 19.30 hours daily, and the refusals were weighted at 07.30 the following day to calculate DMI of each calf. Individual samples of feed offered and refusals were taken every 5 d, and faeces were collected from the rectum at 07.00, 13.00, 19.00 and 01.00 hours daily on days 54–57. Samples of feed, refusals and faeces were dried at 60 °C to a constant weight, ground to pass a 1-mm screen (Wiley mill; Qingdao Ruixintai Instrument Co., Ltd.) and then pooled by calve. These samples were analysed for DM and organic matter (OM; method 942.05), crude protein (method 990.03) and acid-detergent fibre (ADF; method 973.18) based on the procedures of AOAC[18]. Heat stable α-amylase and sodium sulphite were used in the assay of neutral-detergent fibre (NDF)[19]. Acid-insoluble ash was used as an endogenous indicator in the determination of nutrient apparent digestibility and measured according to Van-Keulen and Young[20]. Dietary Co and folate were measured based on the method of AOAC[18] and Alaburda *et al.*[21], respectively. Individual samples of ruminal fluid (150 ml) were obtained via the oesophagus using a stomach tube[22] at 06.30, 12.30, 18.30 and 00.30 hours daily on days 58 and 59. To avoid the contamination of saliva, the first obtained 200 ml of fluid was discarded. Samples of rumen fluid were determined for pH (Sartorius Basic pH Meter PB-10, Sartorius AG) and then strained using four layers of medical gauze. A 5-ml strained fluid was acidified with 1 ml H_2SO_4 (20 g/l) to measure ammonia N according to AOAC[18]. Another 5-ml strained fluid was deproteinised with 1 ml meta-phosphoric acid (250 g/l) for the analysis of VFA by GC (Trace 1300; Thermo Fisher Scientific Co., Ltd.) using 2-ethylbutyric acid as an internal standard[23]. Further 15-ml strained fluid was sonicated at 4 °C and 20 s pulse rate for 10 min, centrifuged at 4 °C and 25 000 ***g*** for 15 min and separated the supernatant to determine enzyme activities based on the procedures of Agarwal *et al.*[24]. All these samples above were stored at –20 °C until analysis. Additional 5-ml strained fluid was stored at –80 °C for the extraction of microbial DNA. These samples of rumen fluid from different time were mixed in equal proportions by each calf. Microbial DNA was extracted by the RBB + C method from 1·0 ml homogenised ruminal fluid[25]. The quality and quantity of extracted DNA were checked by agarose gel electrophoresis and spectrophotometer (Thermo Scientific), respectively. The primer sequences of target microbes are described in Table 2. The sample-derived DNA standard for each qPCR assay was generated from the treatment pool set of microbial DNA using the regular PCR. The PCR products were purified using the MiniBest DNA Fragment Purification on Kit Ver.4.0 (Takara Biotechnology Co., Ltd.) and quantified by a spectrophotometer. The copy number concentration of each standard was calculated according to the length of the PCR product and the mass concentration. Tenfold serial dilutions were used for establishing standard curves of targeted microbes[26]. Amplification and detection of qPCR were carried out in a StepOne™ system (Thermo Fisher Scientific Co., Ltd.). Samples were assayed in triplicate. The reaction mixture (20 μl) included

Table 2. PCR primers for real-time PCR assay

Target species	Primer sequence (5'–3')	GenBank accession no.	Size (bp)
Total bacteria	F: CGGCAACGAGCGCAACCC R: CCATTGTAGCACGTGTGTAGCC	CP058023.1	147
Total anaerobic fungi	F: GAGGAAGTAAAAGTCGTAACAAGGTTTC R: CAAATTCACAAAGGGTAGGATGATT	GQ355327.1	120
Total protozoa	F: GCTTTCGWTGGTAGTGTATT R: CTTGCCCTCYAATCGTWCT	HM212038.1	234
Total methanogens	F: TTCGGTGGATCDCARAGRGC R: GBARGTCGWAWCCGTAGAATCC	GQ339873.1	160
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
P. ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG R: CATCCTATAGCGGTAAACCTTTGG	LT975683.1	74
Rb. amylophilus	F: CTGGGGAGCTGCCTGAATG R: GCATCTGAATGCGACTGGTTG	MH708240.1	102

10 μl SYBR Premix Ex TaqTMII (TaKaRa Biotechnology Co., Ltd), 2 μl template DNA, 0·8 μl of each primer, 0·4 μl ROX Reference Dye II and 6·0 μl double standard sterile water. The conditions were 1 cycle of 50 °C for 2 min and 95 °C for 2 min for initial denaturation, followed by 45 cycles of 95 °C for 15 s, at annealing temperature for 1 min, and then product elongation at 72 °C for 30 s. Specificity of amplification was performed via dissociation curve analysis of PCR end products by increasing the temperature at a rate of 1 °C every 30 s from 60 °C to 95 °C.

Individual blood samples were collected by the coccygeal vessel at 10.30 hours on day 60 using 10 ml evacuated tubes (Jiancheng Biological Engineering Co., Ltd), centrifuged at 2500 ***g*** and 4 °C for 10 min to separate serum, and then stored at −20 °C. Serum glucose, albumin, total protein, Hcy and folate were measured by the Infinite F50 Microplate reader (Tecan Austria GmbH) with ELISA kits (Shanghai Meilian Biology Science and Technology Co., Ltd). Serum vitamin B_{12} was analysed using the HPLC (Agilent 1100 VWD) according to the method of Hasnat *et al.*[27].

Calculation and statistical analyses

The feed conversion ratio for each calf was calculated as daily DMI divided by ADG. Data for DMI were firstly averaged by every 30 d, and then data for DMI, ADG and feed conversion ratio were analysed by the mixed procedure of SAS (Proc Mixed; SAS, 2002)[28] with a 2 (FA addition) × 2 ($CoSO_4$ addition) completely randomised block design, the model as follows:

$$Yijklm = \mu + Bi + F_j + C_k + (\mathrm{FC})_{jk} + T_l + (\mathrm{TF})_{jl} + (\mathrm{TC})_{kl} + (\mathrm{TFC})_{jkl} + R_{m:ijk} + \varepsilon_{ijklm}$$

Other measurements were analysed using the model:

$$Yijklm = \mu + Bi + F_j + C_k + (\mathrm{FC})_{jk} + R_{m:ijk} + \varepsilon_{ijkm}$$

where $Yijklm$ is the dependent variable, μ is the overall mean, B_i is the random effects of the ith block, F_j is the fixed effects of FA addition (j = with or without), C_k is the fixed effects of $CoSO_4$ addition (k = with or without), $(FC)_{jk}$ is the FA × $CoSO_4$ interaction, T_l is the fixed effect of time, $(TF)_{jl}$ is the time × FA interaction, $(TC)_{kl}$ is the time × $CoSO_4$ interaction, $(TFC)_{jkl}$ is the time × FA × $CoSO_4$ interaction, R_m is the random effects of the mth calf and ϵ_{ijklm} is the residual error. Initial measures were used as a covariate to improve identifying effects associated with dietary treatment of Co and FA. Mean separations using probability of difference tests (PDIFF in SAS) were conducted only for effects that were significant at $P < 0{\cdot}050$. Significant differences were declared at $P < 0{\cdot}050$.

Results

Performance

As shown in Table 3, the significant FA and Co interaction was observed for BW of 60 d and ADG which increased with FA or Co supplementation, but the increased magnitude was greater when Co was supplemented with diets without FA addition compared to diets with FA addition. DMI of calves increased with Co or FA inclusion. The BW of calves were similar among four groups at the beginning of the trial and were increased by Co supplementation during the trial. Feed conversion ratio reduced with Co supplementation but was unchanged with FA inclusion.

Digestibility and rumen fermentation

There was no significant FA × Co interaction for total-tract nutrient digestibility and rumen fermentation parameters (Table 4). Digestibility of DM, OM, crude protein, NDF and ADF increased for FA or Co addition. Rumen pH was not affected by FA but reduced with Co supplementation. Ruminal total VFA concentration was elevated with the inclusion of FA or Co.

Table 3. Effects of folic acid (FA) and cobalt sulphate ($CoSO_4$) addition on DM intake (DMI), average daily gain (ADG) and feed conversion ratio (FCR) in male calf studies groups*
(Mean values with their standard errors of the mean)

	FA−†		FA+			*P*‡		
Item	Co−	Co+	Co−	Co+	SEM	FA	Co	FA × Co
DMI (kg/d)	3·84	4·23	4·26	4·44	0·069	0·044	0·045	0·378
Body weight (kg)								
0 d	109	110	110	108	2·04	0·578	0·662	0·704
30 d	139	147	144	144	2·25	0·326	0·027	0·084
60 d	166	181	178	179	2·94	0·017	0·019	0·028
ADG (kg/d)	0·96	1·18	1·14	1·20	0·024	0·048	0·008	0·041
FCR (kg/kg)	4·01	3·58	3·74	3·71	0·066	0·222	0·040	0·088

* The *P* value of time for DMI, ADG and FCR was 0·005, 0·081 and 0·019. The time × FA, time × Co and time × FA × Co interaction for all the studied variables were not significant ($P > 0·05$).
† FA−, without FA; FA+, with 7·2 mg FA/kg DM; Co−, without Co; Co+, with 0·11 mg Co/kg DM as cobalt sulphate.
‡ FA: FA + *v.* FA−; Co: Co+ *v.* Co−; FA × Co: the interaction between FA and Co addition.

Table 4. Effects of folic acid (FA) and cobalt sulphate ($CoSO_4$) addition on total-tract nutrient digestibility and ruminal fermentation in male calf studies groups*
(Mean values with their standard errors of the mean)

	FA−†		FA+			*P*‡		
Item	Co−	Co+	Co−	Co+	SEM	FA	Co	FA × Co
Digestibility (%)								
DM	60·2	61·7	61·7	63·7	0·41	0·034	0·034	0·745
Organic matter	63·1	64·2	64·1	66·1	0·19	0·006	0004	0·381
Crude protein	64·3	65·6	66·6	68·6	0·23	0·001	0·003	0·456
Neutral-detergent fibre	56·3	58·1	57·4	60·9	0·31	0·001	0·001	0·107
Acid-detergent fibre	52·6	53·9	53·4	55·8	0·33	0·031	0·005	0·354
Ruminal fermentation								
pH	6·78	6·68	6·73	6·62	0·023	0·201	0·024	0·887
Total VFA (mM)	98·3	104	102	109	0·99	0·036	0·003	0·856
Mol/100 mol								
Acetate	66·1	63·6	68·0	64·5	0·22	0·031	0·022	0·712
Propionate	18·8	20·7	17·3	19·7	0·21	0·263	0·019	0·410
Butyrate	9·18	9·21	8·97	9·63	0·124	0·316	0·041	0·629
Valerate	2·23	2·39	2·32	2·57	0·069	0·133	0·214	0·966
Isobutyrate	1·18	1·27	1·09	1·13	0·031	0·812	0·330	0·225
Isovalerate	2·51	2·63	2·32	2·57	0·016	0·910	0·708	0·335
A:P	3·53	3·07	3·93	3·26	0·043	0·039	0·030	0·420
Ammonia N (mg/100 ml)	13·4	12·5	12·6	12·0	0·45	0·447	0·386	0·851

* The *P* value of time for digestibility of CP, NDF and ADF was 0·008, 0·011 and 0·020. The *P* value of time for other variables, time × FA, time × Co and time × FA × Co interaction for all the studied variables was not significant ($P > 0·05$).
† FA−, without FA; FA+, with 7·2 mg FA/kg DM; Co−, without Co; Co+, with 0·11 mg Co/kg DM as cobalt sulphate.
‡ FA: FA + *v.* FA−; Co: Co+ *v.* Co−; FA × Co: the interaction between FA and Co addition.
A:P was the ratio of acetate to propionate.

Supplementing FA in diets did not affect propionate percentage but increased acetate percentage and the ratio of acetate to propionate. Dietary Co inclusion elevated propionate percentage and reduced acetate percentage and acetate to propionate ratio. Butyrate percentage was unaltered with FA inclusion but elevated by Co addition. Percentages of valerate, isobutyrate and isovalerate as well as concentration of ammonia N were not influenced by treatments.

Rumen enzyme activity and microbial population

Rumen enzyme activity and microbial population were summarised in Table 5. Significant FA × Co interaction was not observed. Activities of carboxymethyl-cellulase and pectinase increased with FA inclusion but were not influenced by Co supplementation. Dietary inclusion of FA or Co did not affect activities of cellobiase and protease but increased activities of xylanase and α-amylase. Dietary inclusion of FA or Co increased populations of total bacteria, fungi, protozoa, *R. albus*, *Fibrobacter succinogenes* and *P. ruminicola* but did not influence populations of *Butyrivibrio fibrisolvens* and *Ruminobacter amylophilus*. Total methanogens population was unchanged with FA inclusion and decreased with Co supplementation. In contrast, *R. flavefaciens* population increased with FA inclusion and was unchanged with Co supplementation.

Blood metabolites

Blood metabolites were shown in Table 6; there was no significant FA and Co interaction for blood metabolites in calves. Dietary inclusion FA did not influence concentrations of blood glucose, total protein, albumin and vitamin B_{12}, but increased folates and reduced Hcy. Dietary inclusion of Co increased

Table 5 Effects of folic acid (FA) and cobalt sulphate ($CoSO_4$) addition on ruminal microbial enzyme activity and microflora in male calf studies groups* (Mean values with their standard errors of the mean)

	FA–†		FA+			*P*‡		
Item	Co–	Co+	Co–	Co+	SEM	FA	Co	FA × Co
Microbial enzyme activity								
Carboxymethyl-cellulase	0·14	0·15	0·18	0·20	0·014	0·023	0·263	0·842
Cellobiase	0·13	0·15	0·14	0·16	0·013	0·110	0·324	0·966
Xylanase	0·78	0·85	0·88	0·92	0·022	0·012	0·015	0·875
Pectinase	1·44	1·47	1·51	1·53	0·014	0·031	0·252	0·939
α-amylase	1·79	1·88	1·84	1·98	0·024	0·037	0·012	0·750
Protease	0·74	0·78	0·79	0·84	0·042	0·209	0·238	0·345
Microflora (copies/ml)								
Total bacteria, $\times 10^{11}$	7·21	7·99	8·06	8·85	0·267	0·033	0·031	0·946
Total anaerobic fungi, $\times 10^{7}$	3·78	4·37	4·36	5·07	0·242	0·047	0·046	0·950
Total protozoa, $\times 10^{5}$	2·18	2·60	2·39	3·33	0·226	0·035	0·015	0·574
Total methanogens, $\times 10^{9}$	8·94	7·42	8·79	7·76	0·190	0·243	0·003	0·519
R. albus, $\times 10^{8}$	3·39	4·88	4·54	5·44	0·341	0·048	0·033	0·820
R. flavefaciens, $\times 10^{9}$	2·50	2·96	2·87	3·20	0·346	0·639	0·563	0·921
F. succinogenes, $\times 10^{10}$	5·86	7·08	6·80	8·67	0·580	0·028	0·020	0·782
B. fibrisolvens, $\times 10^{9}$	2·18	2·54	2·55	2·94	0·221	0·400	0·418	0·969
P. ruminicola, $\times 10^{9}$	3·99	4·57	4·59	5·79	0·163	0·010	0·011	0·354
Rb. amylophilus, $\times 10^{8}$	1·66	1·40	1·43	1·47	0·124	0·728	0·663	0·554

* The *P* value of time, time × FA, time × Co and time × FA × Co interaction for all the studied variables was not significant ($P > 0·05$).
‡ FA–, without FA; FA+, with 7·2 mg FA/kg DM; Co–, without Co; Co+, with 0·11 mg Co/kg DM as cobalt sulphate.
† FA: FA + *v.* FA–; Co: Co+ *v.* Co–; FA × Co: the interaction between FA and Co 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), α-amylase (μmol glucose/min per ml) and protease (μg hydrolysed protein/min per ml).

Table 6 Effects of folic acid (FA) and cobalt sulphate ($CoSO_4$) addition on blood metabolites in male calf studies groups (Mean values with their standard errors of the mean)

	FA–*		FA+			*P*†		
Item	Co–	Co+	Co–	Co+	SEM	FA	Co	FA × Co
Glucose (μmol/l)	307	363	320	370	11·1	0·978	0·036	0·991
Total protein (μg/ml)	882	835	817	865	27·8	0·756	0·990	0·413
Albumin (μg/ml)	334	316	310	314	10·4	0·183	0·389	0·225
Folates (μmol/l)	13·2	12·7	21·3	24·7	2·31	0·003	0·874	0·374
Homocysteine (μmol/l)	11·3	9·41	7·38	8·69	0·354	0·016	0·713	0·341
Vitamin B_{12} (ng/ml)	1·78	2·39	1·46	1·98	0·266	0·267	0·034	0·857

* FA–, without FA; FA+, with 7·2 mg FA/kg DM; Co–, without Co; Co+, with 0·11 mg Co/kg DM as cobalt sulphate.
† FA: FA + *v.* FA–; Co: Co+ *v.* Co–; FA × Co: the interaction between FA and Co addition.

concentrations of glucose and vitamin B_{12} but did not influence total protein, albumin, folates and Hcy.

Discussion

That supplementing Co at 0·11 mg/kg DM in diets including 0·09 mg Co/kg DM increased DMI of male calves was in agreement with Schwarz *et al.*[6], in which feed intake of growing bulls increased when dietary Co level increased from 0·07 to 0·20 mg/kg. The increase in DMI was a reason for the increase in ADG and was probably due to an increase in blood propionate clearance rate with Co addition[3]. Blood propionate concentration was negatively related to feed intake[29]. Vitamin B_{12}, as a cofactor of methylmalonyl-CoA mutases, is involved in the entry of propionate into the Krebs cycle for providing energy or being used as a gluconeogenesis substrate[2]. Marston *et al.*[30] reported that low level of dietary Co impaired propionate metabolism, causing the remove rate of blood propionate to reduce. Likewise, studies in finishing steers observed increased DMI and ADG when supplementing 0·10 or 0·15 mg Co/kg DM in diets containing 0·04 mg Co/kg DM[3,4]. The increase in total-tract nutrient digestibility in calves receiving 0·11 mg Co/kg DM addition showed a stimulatory impact of Co or vitamin B_{12} on nutrient digestion and was another reason for the increase in ADG. Dietary Co is essential for rumen microbial vitamin B_{12} synthesis[1]. Moreover, the divalent Co cations from $CoSO_4$ could form a bridge between microbes and feed particles which are negatively charged[31]. Therefore, dietary Co inclusion promoted feed degradation as reflected by the increase in rumen total VFA concentration. Furthermore, the positive response of nutrient digestibility was likely associated with an increase in rumen vitamin B_{12} synthesis as reflected by the higher blood B_{12} concentration for calves receiving Co supplementation. Studies indicated that Co supplementation increased rumen and plasma vitamin B_{12} concentrations in steers[3,4], and that digestibility of DM, OM and crude protein increased with sub-cutaneous injection of

vitamin B_{12} in goats[32]. Similar to the present study, Wang *et al.*[7] found increased apparent digestibility of DM, OM, crude protein, NDF and ADF with dietary supplementation of 0·25, 0·50 or 0·75 mg Co/kg DM in lambs. The increase in rumen total VFA concentration and propionate molar percentage was in line with the increase in α-amylase activity and populations of total bacteria, fungi, protozoa and *P. ruminicola*. The decrease in acetate to propionate ratio suggested that rumen fermentation mode was altered to more propionate formation and should be the reason for the decrease in methanogens population and increase in blood glucose content with Co supplementation. The increment of propionate production increased the substrate for gluconeogenesis but caused a decrease in rumen hydrogen which is required by methanogens to synthesise methane[2,33]. These results suggested that dietary Co inclusion was required for the growth of microbes responsible for non-structural carbohydrates digestion and propionate production. It has been demonstrated that vitamin B_{12} participates in bacteria DNA synthesis and propionate production[3]. Likewise, some studies showed that vitamin B_{12} supplementation stimulated the growth and propionate production of some strains of *P. ruminicola in vitro*.[8,9], and that dietary Co inclusion decreased acetate to propionate ratio and increased propionate proportion in steers[3,4]. However, others reported that molar percentages of SCFA were not influenced by increasing dietary Co level from 0·17 to 0·29 mg/kg in cows[34] or from 0·09 to 0·14 mg/kg in growing steers[4]. Since the amount of rumen vitamin B_{12} synthesis depended on levels of Co, sugars and NDF in diets[3,35], the divergent responses of rumen fermentation parameters to Co supplementation were likely due to the differences in diets and Co level in these studies.

In accordance with the results of Wang *et al.*[13], the increase in DMI and ADG was found with dietary FA inclusion in calves. The change of ADG could be ascribed to the increment of DMI and nutrient digestibility with FA inclusion. In addition, results that blood folates increased and Hcy decreased suggested that FA inclusion probably promoted the conversion of Hcy to methionine, and this should be another reason of the increase in ADG. FA, in the form of 5-methyl-THF, donors a methyl group to Hcy to regenerate methionine, playing a crucial role in protein synthesis metabolism[10]. Studies with cows reported that B vitamins addition enhanced protein metabolic efficiency, resulting in an increase in milk performance[36], and that FA and vitamin B_{12} supplementation increased methionine utilisation for protein synthesis[16]. Moreover, others observed increased weight gain in calves with intramuscular injections of FA[37]. The elevation of total-tract digestibility of DM and OM was in accordance with the results of Wang *et al.*[13] and Liu *et al.*[14] in calves, suggesting that both ruminal and post-ruminal nutrient digestion might be promoted by FA inclusion. The changes of rumen total VFA concentration showed a stimulatory effect of FA on nutrient degradation. Furthermore, FA is required for the growth and digestive juices secretion of pancreatic cells[38]. Parnian-Khajehdizaj *et al.*[15] observed that post-ruminal and total-tract DM digestibility increased with FA supplementation *in vitro*. The increment in rumen acetate percentage and acetate to propionate ratio was in accordance with the changes of total-tract digestibility of NDF and ADF and was associated with the increase in activities of carboxymethyl-cellulase, pectinase and xylanase and populations of total bacteria, fungi, protozoa, *R. flavefaciens*, *R. albus*, *F. succinogenes* and *P. ruminicola* for FA inclusion. Anaerobic fungi yields fibre-degrading enzyme and can penetrate into plant tissues unaccessible for bacteria[39,40]. Protozoa is responsible for approximately 30 % of fibre degradation and a synergistic interaction existed between *R. flavefaciens*, *F. succinogenes* and *P. ruminicola* in cellulose digestion[39,40]. Therefore, the present results showed that FA provision stimulated rumen microbial growth, causing fibre digestion to increase and rumen fermentation to alter to more acetate production. These results should be related to the functions of FA in the one-carbon metabolism. FA, in the form of 5,10-methylene-THF, 10-formyl-THF and 5-methyl-THF, provides one-carbon units for thymidylate synthesis, purine synthesis and methylation reactions and is essential for cell division and protein synthesis[10]. Likewise, early studies of *in vitro* reported that *R. flavefaciens* required THF or 5-methyl-THF for maximum growth[12], and that rumen cellulose digestion was stimulated by FA[11]. Recent studies found that total-tract NDF and ADF digestibility, rumen acetate production and fibrolytic microbial populations increased with FA inclusion in weaned calves[13,14].

Supplementary Co is used by microbes to synthesise vitamin B_{12}, and vitamin B_{12} dependent methionine synthase is essential for the regeneration of methionine and THF, a biologically active form of folates[1,41]. Similar BW of 60 d and ADG were observed for calves with addition of Co, FA as well as Co and FA together, but the interaction of FA and Co for nutrient digestibility and ruminal fermentation parameters was not significant. The results suggested that supplementing Co in the FA+ diets probably did not increase the utilisation efficiency of FA. Likewise, Graulet *et al.*[17] found that milk yield was similar for dairy cows receiving FA addition alone or FA plus vitamin B_{12} addition.

Conclusions

Dietary FA or Co inclusion increased ADG, nutrient digestibility and rumen VFA production in calves. Addition of FA stimulated rumen cellulolytic microbial growth, resulting in an increase in fibre digestion and acetate production. Supplemented Co was mainly used by microbes responsible for non-structural carbohydrates digestion and propionate production. Supplementing 0·11 mg Co/kg DM in calf diets containing 0·09 mg Co/kg DM probably did not increase FA utilisation efficiency, since no further increase in ADG was observed with FA and Co supplementation compared with FA or Co addition alone.

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

The authors declare that no conflict of interest exists.

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