British Journal of Nutrition (2021), 126, 1288–1295 doi:10.1017/S0007114520005243

Effects of riboflavin supplementation on performance, nutrient digestion, rumen microbiota composition and activities of Holstein bulls

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(Submitted 20 October 2020 – Final revision received 20 December 2020 – Accepted 22 December 2020 – First published online 8 January 2021)

Abstract

To investigate the influences of dietary riboflavin (RF) addition on nutrient digestion and rumen fermentation, eight rumen cannulated Holstein bulls were randomly allocated into four treatments in a repeated 4 × 4 Latin square design. Daily addition level of RF for each bull in control, low RF, medium RF and high RF was 0, 300, 600 and 900 mg, respectively. Increasing the addition level of RF, DM intake was not affected, average daily gain tended to be increased linearly and feed conversion ratio decreased linearly. Total tract digestibilities of DM, organic matter, crude protein (CP) and neutral-detergent fibre (NDF) increased linearly. Rumen pH decreased quadratically, and total volatile fatty acids (VFA) increased quadratically. Acetate molar percentage and acetate:propionate ratio increased linearly, but propionate molar percentage and ammonia-N content decreased linearly. Rumen effective degradability of DM increased linearly, NDF increased quadratically but CP was unaltered. Activity of cellulase and populations of total bacteria, protozoa, fungi, dominant cellulolytic bacteria, *Prevotella ruminicola* and *Ruminobacter amylophilus* increased linearly. Linear increase was observed for urinary total purine derivatives excretion. The data suggested that dietary RF addition was essential for rumen microbial growth, and no further increase in performance and rumen total VFA concentration was observed when increasing RF level from 600 to 900 mg/d in dairy bulls.

Key words: **Riboflavin: Nutrient degradation: Rumen fermentation: Microbiota: Urinary purine derivatives: Holstein bulls**

Early research considered that the amount of B vitamins synthesised by rumen microbes could meet the requirement of ruminants and that vitamins synthesised by some bacteria species could meet the requirements of other species by cross-feeding in the rumen[1]. However, Van Gylswyk *et al.* found that the supply of certain B vitamins from rumen microbial synthesis was too low to meet the optimal growth of some species[2]. Recent studies observed that dietary addition of folic acid or pantothenate increased concentration of rumen total volatile fatty acids (VFA), abundance of cellulolytic bacteria and degradabilities of DM, neutral-detergent fibre (NDF) and crude protein (CP) in steers[3,4].

Riboflavin (RF) is required by all organisms. In the form of flavin mononucleotide and flavin adenine dinucleotide, RF functions in electron transfer reactions related to energy, carbohydrate, lipid and amino acid metabolism[5]. Flavin coenzyme is required for the activation and metabolism of other B vitamins, such as folate, cyanocobalamin and pyridoxine[5]. Rumen microbial RF synthesis was associated with dietary composition and increased with increasing the level of readily degradable carbohydrates and protein[6,7]. Apparent ruminal synthesis of RF calculated as the duodenal flow minus its daily intake was negative in cows receiving diets, such as high fibre and low N, alfalfa or orchardgrass silages as the sole forage or different types of dry maize grain[6–8]. Santschi *et al.* noted 99·3 % RF supplemented in diets disappeared before the duodenal cannula of cows[9]. These results suggested that rumen microbes might need to use dietary RF to support their growth and nutrient digestion. Studies *in vitro* reported that RF was essential for the growth of *Ruminococcus flavefaciens*[10] and *R. albus*[11] and that the digestion of cellulose by rumen microorganisms was stimulated by RF addition[12]. However, there was no reports about the impacts of dietary RF supplementation on ruminal fermentation and bacterial growth *in vivo*.

On account of the results above, it was speculated that dietary RF supplementation could promote rumen microbial growth and nutrients degradation. Therefore, the objective of current study was to investigate the influences of RF supplementation on ruminal fermentation, nutrients degradation, microbial abundance and urinary excretion of purine derivatives in Holstein bulls.

Materials and methods

Animals and experimental design

The experiment was approved by the Animal Care and Use Committee of Shanxi Agriculture University and conducted at Shanxi Agriculture University experiment station. Eight Holstein

Abbreviation: CP, crude protein; ED, effective degradability; NDF, neutral-detergent fibre; RF, riboflavin; VFA, volatile fatty acids.

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British Journal of Nutrition

dairy bulls (24 (SD 3·4) months of age and 614 (SD 28·9) kg body weight) equipped with a ruminal cannula were randomly divided into four treatments in a repeated 4 × 4 Latin square design with a 4 × 24 d period. The first 14 d of each period were for adaptation and the followed d 15–24 were for data and sample collection. Bulls in control, low RF, medium RF and high RF groups were individually offered basal diets supplemented with RF 0, 300, 600 and 900 mg/d, respectively. Basal diets were formulated according to the National Research Council[13] to meet nutrient requirement of dairy bulls (Table 1). Content of RF in the basal diets was 4·2 mg/kg DM and was measured according to the method described by Santschi *et al.*[9]. Supplementary RF (feed grade, 980 mg RF/g; Guangzhou Yeshang trade Co. Ltd) was mixed into the premix and then mixed with the first third of morning ration before daily feeding. The low supplementation level of RF was estimated as: (RF net requirement – rumen synthesised RF × intestinal absorptivity)/intestinal absorptivity. Daily estimated net requirement of RF for tissues in dairy cow of 650 kg body weight was 95 mg/d, estimated apparent ruminal synthesis of RF was 15·2 mg/kg of digestible organic matter consumed per d and estimated intestinal absorption percentage of RF was 23–25 %[13]. All bulls were individually housed in a stall of 2·5 × 3 m, fed at 07.30 and 19.30 hours daily and had free access to clean drinking water.

Data collection and sampling procedures

All bulls were weighed individually at the start and every 24 d of the trial to determine changes in body weight. During each data and sample collection period, feed offered and refused for each bull were quantified and sampled daily. Total faeces and urine voided by individual bull in 24 h were gathered by using a harness system fitted with a faecal collection bag and urine collection aprons, weighed and sampled. Faecal sample, a representative of 1/12 daily faecal production, was blended with 10 % tartaric acid solution according to 1/4 of sample wet weight. Urine sample, 1·0 % of daily urine excretion, was put into bottles containing 10 % sulphuric acid solution to ensure pH below 3·0. Feed (offered and refused), faeces and urine samples were stored at −20°C. At the end of the trial, all of the feed and faeces samples were dried (55°C and 48 h), mixed by bull and period and then ground to pass through a 1-mm sieve screen for chemical analysis.

Rumen fluid was sampled on days 23 and 24 of each period. Samples of 200 ml were collected from several sites of the rumen (reticulum, dorsal and ventral sac) in the same proportion at 0, 3, 6, 9 and 12 h after the morning feeding. Ten ruminal samples for each bull per period were collected and used for chemical analysis. Rumen fluid pH was measured using a portable pH meter (PHS-3C, Shanghai Meiyingpu Instrument Manufacturing Co. Ltd) and then filtered using four layers of medical gauze. Filtrate of 5 ml was mixed with metaphosphoric acid (1 ml, 250 g/l) or H_2SO_4 (1 ml, 20 g/l) and frozen at −20°C for subsequent analyses of VFA and ammonia-N, respectively. Filtrate of 50 ml was placed in liquid N_2 and kept at −80°C for analyses of microbial abundance and enzyme activity, respectively.

Table 1. Ingredient and chemical composition of the basal diet

Ingredients	Contents (g/kg DM)
Maize silage	430
Alfalfa hay	70
Maize grain, ground	234
Wheat bran	40
Soyabean meal	30
Rapeseed meal	40
Cottonseed cake	130
Calcium carbonate	13
Salt	5
Calcium phosphate	3
Mineral and vitamin premix*	5
Chemical composition	
Organic matter	895
Crude protein	146
Ether extract	23·6
Neutral-detergent fibre	387
Acid-detergent fibre	241
Non-fibre carbohydrate†	340
Ca	7·6
P	4·2

* Contained per kg premix: 1600 mg Cu, 8000 mg Mn, 7500 mg Zn, 120 mg iodine, 20 mg Co, 1640 mg vitamin A, 600 mg vitamin D and 200 mg vitamin E.
† Non-fibre carbohydrate, calculated by 1000 – crude protein – neutral-detergent fibre – fat – ash.

In situ *nutrient degradability*

Nutrient degradability of maize silage and concentrate was measured *in situ* according to the Agricultural and Food Research Council[14]. Samples of the air-dried maize silage and concentrate were ground to pass through a 2·5-mm screen. On day 15 of each period, 3·0 g of maize silage and 3·5 g of concentrate were incubated separately in nylon bags (8 × 12 cm, pore size 47 μm) suspended in the rumen of each dairy bull. The duplicated bags were inserted at 2 h after feeding, taken out at 0, 4, 8, 12, 24, 36, 48 and 72 h, washed with cold water and then dried at 65°C for 24 h. Kinetic parameters of DM, CP and NDF degradabilities were calculated using the non-linear regression procedure of SAS[15]. Nutrient disappearance percentage at each incubation time of individual bull was calculated by using the equation:template:bgmath

$$y = a + b\left(1 - e^{-c(t-L)}\right) \text{ for } t > L \text{ and } y = a \text{ when } t < L$$

in which y is the fraction degraded in the time t, a is the soluble fraction, b is the slowly degradable fraction and c is the fractional degradation rate constant at which b is degraded, L (h) is the lag time and t (h) is the time of incubation[16]. Effective degradability (ED) was calculated as $ED = a + [bc/(c + k)] \times e^{-k \times L}$, where k is the particulate passage rate, which is 0·058 h^{-1} and 0·025 h^{-1} for concentrate and maize silage, respectively[16].

Chemical analyses

Feeds, refusals and faeces samples were analysed for DM (method 920.36) by drying at 135°C for 3 h, CP (method 984.13) by a Kjeldahl method and ash (method 923.03) by combustion at 550°C for 3 h[17]. Organic matter was estimated as DM minus ash. NDF was determined according to the method of Van Soest *et al.*[18], and heat α-amylase and Na_2SO_3 were used in the

procedure. Acid-detergent fibre was measured according to the Association of Official Analytical Chemists (method 973.18)[17]. Allantoin and uric acid were determined using DR3900 spectrophotometer (Shanghai Zhenbao Electromechanical Equipment Co. Ltd) according to the method of the International Atomic Energy Agency[19]. Rumen VFA was analysed using GC (GC7890; Shandong Jinpu Analytical Instrument Co. Ltd), and ammonia-N was done by a method of the Association of Official Analytical Chemists[17]. Microbial enzyme activities in the rumen fluid were measured using the methods of Agarwal *et al.*[20].

Extraction of microbial DNA and real-time PCR

Isolation of microbial DNA was performed by using the repeated bead-beating method[21] from 1·5 ml of homogenised ruminal fluid. The integrity and purity of extracted DNA were checked via agarose gel electrophoresis and NanoDrop 2000 Spectrophotometer (Thermo Scientific, NanoDrop Technologies), respectively. The target microbial primers set sequences are shown in Table 2. For absolutely quantifying the copy number of gene, nine sample-derived DNA standards were prepared by using the regular PCR. The PCR products were purified using the PureLinkTM Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific Co. Ltd) and quantified using a spectrophotometer. Copy number of each sample-derived standard was evaluated according to the PCR product length and mass concentration. The target DNA was quantified by using ten-fold serial dilutions from 10^1 to 10^8 DNA copies[22]. The quantitative PCR assay was carried out in a StepOne™ system (Bio-Rad) in triplicate. The reaction mixture (20 µl) contained 10 µl SYBR Premix Taq™ II (TaKaRa), 2 µl DNA template, 0·8 µl of each primer (10 µmol/µl), 0·4 µl ROX Reference Dye II (TaKaRa) and 6·0 µl nuclease-free water. The conditions of quantitative PCR assay were as follows: one cycle at 50°C for 2 min and 95°C for 2 min for initial denaturation, followed by forty cycles at 95°C for 15 s, and then at annealing temperature for 30 s and extension at 60° C for 1 min.

Statistical analyses

Feed conversion ratio for each bull was estimated as DM intake divided by average daily gain. Data were analysed by the mixed model procedure of SAS (Proc Mixed; SAS 2002)[15] with a repeated 4 × 4 Latin square design to account for effects of square, period within square, bull within square and treatment. The treatment was considered as a fixed effect; square, period within square and bull within square were considered as random effects. Data for ruminal pH, VFA, ammonia-N, microbial enzyme activity and microbiota were summarised by sampling time and then analysed using the same mixed model but with time included as a repeated measure using compound symmetry. Rumen samples were analysed using the average per sampling time. Linear and quadratic orthogonal contrasts were tested using the CONTRAST statement of SAS with coefficients estimated based on the level of RF supplementation. The significant effects for the factors were suggested at $P < 0{\cdot}05$, and trends were declared at $0{\cdot}05 < P < 0{\cdot}10$.

Results

Growth performance, nutrient apparent digestibility and ruminal fermentation

Dietary addition of RF did not affect DM intake of bulls (Table 3). No significant differences were observed for body weight among treatments at the beginning or at the end of the trial. Average daily gain tended to be linearly increased ($P = 0{\cdot}082$), and feed conversion ratio was linearly decreased ($P = 0{\cdot}039$) with increasing addition level of RF. Increasing supplementation level of RF, total tract digestibilities of DM, organic matter, CP and NDF increased linearly ($P < 0{\cdot}05$) but digestibility of acid-detergent fibre was unchanged. Increasing the level of RF addition, rumen pH decreased quadratically ($P = 0{\cdot}041$), total VFA concentration

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	CP058023.1	147
Total anaerobic fungi	F: AGGAAGTAAAAGTCGTAACAAGGTTTC 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
Ruminobacter amylophilus	F: CTGGGGAGCTGCCTGAATG R: GCATCTGAATGCGACTGGTTG	MH708240.1	102
Prevotella ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG R: CATCCTATAGCGGTAAACCTTTGG	LT975683.1	74

F, forward; R, reverse.

Table 3. Effects of riboflavin (RF) supplementation on DM intake (DMI), average daily gain (ADG), feed conversion ratio (FCR), nutrient digestibility and rumen fermentation in Holstein bulls
(Mean values with their standard errors)

	Treatments*					*P*†	
Item	Control	LRF	MRF	HRF	SEM	Linear	Quadratic
DMI (kg/d)	11·2	10·9	11·3	11·6	0·20	0·35	0·48
Body weight (kg)							
Initial body weight	741	738	745	744	13·7	0·92	0·97
Final body weight	770	768	778	775	13·8	0·86	0·99
ADG (kg/d)	1·41	1·47	1·66	1·55	0·102	0·082	0·13
FCR (kg DMI/kg ADG)	7·90	7·40	6·84	7·50	0·137	0·039	0·091
Digestibility							
DM	0·69	0·71	0·72	0·72	0·006	0·028	0·35
Organic matter	0·72	0·74	0·75	0·75	0·006	0·039	0·36
Crude protein	0·73	0·74	0·76	0·76	0·004	0·032	0·39
Neutral-detergent fibre	0·56	0·58	0·60	0·58	0·007	0·041	0·21
Acid-detergent fibre	0·52	0·53	0·56	0·55	0·018	0·20	0·67
Ruminal fermentation							
pH	6·56	6·34	6·22	6·42	0·056	0·288	0·041
Total VFA (mM)	134	138	143	137	1·35	0·205	0·039
mol/100 mol							
Acetate	65·4	66·3	67·8	66·2	0·34	0·019	0·14
Propionate	18·8	17·4	16·2	17·2	0·28	0·012	0·11
Butyrate	10·1	10·8	10·9	10·6	0·24	0·51	0·32
Valerate	2·00	1·78	1·63	1·88	0·090	0·56	0·22
Isobutyrate	1·22	1·20	1·08	1·25	0·038	0·96	0·25
Isovalerate	2·49	2·50	2·29	2·89	0·13	0·41	0·26
Acetate:propionate	3·48	3·81	4·20	3·90	0·073	0·036	0·11
Ammonia-N (mg/100 ml)	10·4	9·81	8·33	9·31	0·17	0·032	0·36

VFA, volatile fatty acids.
* Control, low RF (LRF), medium RF (MRF) and high RF (HRF) with addition of RF 0, 300, 600 and 900 mg/d, respectively. Values are presented as means (*n* 32).
† The *P* values of time for ruminal pH, total VFA and ammonia-N were 0·034, 0·008 and 0·046, respectively. The *P* values of time for VFA molar percentage and acetate:propionate, and the treatment × time interactions for the rumen variables were not significant ($P > 0·05$).

increased quadratically ($P = 0·039$) but molar proportions of butyrate, isobutyrate, isovalerate and valerate were unaffected. Acetate percentage and acetate:propionate ratio increased linearly ($P < 0·05$), while propionate percentage and ammonia-N concentration decreased linearly ($P < 0·05$).

Rumen nutrient degradation

For concentrate DM, the soluble fraction (a) and slowly degradable fraction (b) were unchanged but the degradation rate (c) and ED increased linearly ($P < 0·05$) with increasing RF addition (Table 4). For concentrate CP, the soluble fraction (a) and slowly degradable fraction (b) decreased linearly ($P = 0·001$), degradation rate (c) increased linearly ($P = 0·001$) but ED was unchanged with increasing RF addition.

For maize silage DM, the soluble fraction (a) and ED increased linearly ($P < 0·05$) but the slowly degradable fraction (b) and degradation rate (c) were not affected by the increased level of RF. For maize silage NDF, the soluble fraction (a) and degradation rate (c) increased linearly ($P < 0·05$), ED increased quadratically ($P = 0·010$) but the slowly degradable fraction (b) was unchanged.

Rumen microbial enzyme and microbiota

Increasing dietary RF addition linearly increased ($P < 0·05$) rumen cellulase activity but did not impact activities of α-amylase and protease (Table 5). Populations of total bacteria, fungi, protozoa, dominant cellulolytic bacteria (*R. albus*, *R. flavefaciens* and *Fibrobacter succinogenes*), *Prevotella ruminicola* and *Ruminobacter amylophilus* linearly increased ($P < 0·05$), but *Butyrivibrio fibrisolvens* was unaltered with increasing RF supplementation.

Urinary excretion of purine derivatives

Urinary excretion of allantoin and total purine derivatives linearly increased ($P < 0·05$) with increasing dietary provision of RF (Table 6). Uric acid excretion was not affected by RF addition.

Discussion

DM intake was unaltered, and so the tendency towards increase in average daily gain should be attributed to the stimulation by supplemented RF to nutrients digestibility in the rumen. Studies in cows indicated that the reason of the increase in performance with B vitamins addition, such as folic acid, vitamin B_{12} and pantothenic acid, was the improvement in metabolic efficiency[23,24]. However, dietary RF would be used or degraded by rumen micro-organisms, and only approximately 1 % of the supplemented RF could reach the small intestine and be absorbed[9]. Therefore, the amount of RF available for absorption was probably not increased by dietary supplementation[9]. Likewise, Majee *et al.* noted DM intake was not affected but milk yield tended to be increased when supplementing a B-vitamin blend including RF in dairy cow diets[25].

The increase in total tract digestibilities of DM, organic matter and NDF was in accordance with the elevation in ruminal degradabilities of DM and NDF, reflecting a stimulatory effect

Table 4. Effects of riboflavin (RF) supplementation on *in situ* ruminal digestion kinetics and effective degradability (ED) of concentrate and maize silage in Holstein bulls
(Mean values with their standard errors)

	Treatments*					*P*	
Item	Control	LRF	MRF	HRF	SEM	Linear	Quadratic
Concentrate							
DM							
a†	22·9	22·7	21·3	19·9	0·81	0·203	0·34
b	66·4	65·3	64·8	65·1	1·078	0·23	0·41
c (h^{-1})	0·030	0·031	0·040	0·053	0·002	0·001	0·11
ED	45·7	45·7	48·0	51·1	0·63	0·027	0·37
Crude protein							
a†	23·5	22·7	18·1	16·9	0·75	0·001	0·65
b	69·6	65·3	62·6	56·7	1·38	0·001	0·62
c (h^{-1})	0·026	0·030	0·043	0·052	0·002	0·001	0·15
ED	45·5	45·1	44·7	43·9	0·43	0·21	0·83
Maize silage							
DM							
a†	10·3	10·1	11·9	13·2	0·34	0·001	0·17
b	77·9	77·0	72·3	72·1	1·25	0·060	0·87
c (h^{-1})	0·017	0·018	0·020	0·018	0·001	0·34	0·45
ED	41·7	43·1	44·4	43·1	0·50	0·023	0·37
Neutral-detergent fibre							
a†	1·04	1·27	1·39	1·74	0·079	0·001	0·55
B	83·7	83·8	80·4	82·7	0·96	0·51	0·59
c (h^{-1})	0·019	0·020	0·023	0·019	0·001	0·045	0·067
ED	37·1	38·2	39·9	36·7	0·45	0·82	0·010

* Control, low RF (LRF), medium RF (MRF) and high RF (HRF) with addition of RF 0, 300, 600 and 900 mg/d, respectively. Values are presented as means (n 32).
† Parameters were calculated from the fitted equation $y = a + b(1 - e^{-c(t-L)})$ for $t > L$, where y = percentage of DM disappearance from the nylon bag at time t, a = soluble fraction, b = slowly degradable fraction, c = fraction rate constant at which b is degraded, L = lag time (h) and t = time of incubation (h). ED was calculated using equation $a + bc/(c + k)$, where $k = 0{\cdot}058\ h^{-1}$ for concentrate and $k = 0{\cdot}025\ h^{-1}$ for maize silage.

Table 5. Effects of riboflavin (RF) supplementation on rumen microbial enzyme activity and microbiota in Holstein bulls
(Mean values with their standard errors)

	Treatments*					*P*†	
Item	Control	LRF	MRF	HRF	SEM	Linear	Quadratic
Microbial enzyme activity‡							
Carboxymethyl cellulase	0·24	0·27	0·32	0·27	0·008	0·017	0·084
Cellobiase	0·33	0·35	0·41	0·36	0·009	0·041	0·12
Xylanase	0·93	1·01	1·11	1·08	0·026	0·014	0·21
Pectinase	0·47	0·51	0·56	0·59	0·019	0·017	0·81
α-Amylase	0·67	0·75	0·77	0·67	0·022	0·95	0·069
Protease	1·55	1·65	1·82	1·59	0·080	0·65	0·32
Microbiota (copies/ml)							
Total bacteria, $\times 10^{11}$	9·68	10·70	11·7	11·3	0·24	0·007	0·18
Total anaerobic fungi, $\times 10^{8}$	6·25	9·47	10·7	12·5	0·65	0·001	0·47
Total protozoa, $\times 10^{8}$	4·85	5·45	5·78	6·46	0·26	0·029	0·92
Ruminococcus albus, $\times 10^{8}$	2·50	4·61	6·33	7·70	0·52	0·001	0·25
Ruminococcus flavefaciens, $\times 10^{9}$	2·41	3·98	5·19	4·82	0·34	0·001	0·51
Fibrobacter succinogenes, $\times 10^{10}$	2·92	4·62	4·92	5·01	0·27	0·002	0·45
Butyrivibrio fibrisolvens, $\times 10^{9}$	12·4	11·9	13·8	13·0	0·65	0·34	0·11
Prevotella ruminicola, $\times 10^{10}$	7·17	9·04	10·2	8·62	0·40	0·047	0·19
Ruminobacter amylophilus, $\times 10^{8}$	0·95	1·99	2·05	2·16	0·17	0·011	0·12

* Control, low RF (LRF), medium RF (MRF) and high RF (HRF) with addition of RF 0, 300, 600 and 900 mg/d, respectively. Values are presented as means (n 32).
† The P values of time for microbial enzyme activity and microbiota were significant ($P < 0{\cdot}05$). The treatment × time interactions for microbial enzyme activity and microbiota were not significant ($P > 0{\cdot}05$).
‡ 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).

of RF supplementation on rumen bacterial growth and enzymatic activity. The decrease of rumen pH was related to the positive effect of RF supplementation on total VFA concentration. When rumen pH was below 6·0, the growth of predominant cellulolytic bacteria and degradation of structural carbohydrate would be inhibited[26]. The lower rumen pH was observed in bulls consuming 600 mg RF/d and was 6·22. The increment in total VFA concentration and acetate percentage was in accordance with the elevation in the soluble fraction, degradation rate and ED of maize silage NDF, indicating that rumen VFA and acetate production as well as the rate and extent of NDF degradation were elevated by RF addition. Noziere *et al.* reported that

Table 6. Effects of riboflavin (RF) supplementation on urinary excretion of purine derivatives (PD) in Holstein bulls (Mean values with their standard errors)

	Treatments*					*P*	
Item	Control	LRF	MRF	HRF	SEM	Linear	Quadratic
Allantoin (mmol/d)	161	179	185	187	3·20	0·001	0·064
Uric acid (mmol/d)	5·95	5·99	6·01	5·97	0·017	0·64	0·31
Total PD (mmol/d)	166	185	191	192	3·21	0·001	0·14

* Control, low RF (LRF), medium RF (MRF) and high RF (HRF) with addition of RF 0, 300, 600 and 900 mg/d, respectively. Values are presented as means (*n* 32).

rumen VFA percentage and concentration could be used to reflect the production of VFA and that rumen acetate level was positively correlated with NDF digestibility[27]. Rumen propionate concentration was calculated as total VFA concentration multiplies propionate molar percentage and was not affected (25·2, 24·1, 23·2 and 23·6 mM for bulls in control, low RF, medium RF and high RF groups, respectively) by RF addition. This was in accordance with the unaffected activity of α-amylase with RF supplementation. That butyrate percentage that was not affected by RF supplementation was in agreement with the unaffected population of *B. fibrisolvens* which degrades cellulose and hemicellulose to butyrate and is the main producer of rumen butyrate[28]. Valerate, isobutyrate and isovalerate are end-products of ruminal true protein degradation[29]. The absence of changes of valerate, isobutyrate and isovalerate percentages was consistent with the unmodified concentrate CP degradability and was likely associated with the unaltered activity of protease with RF supplementation.

The responses of carboxymethyl cellulase, cellobiase, xylanase and pectinase activities were in accordance with the changes in populations for cellulolytic bacteria (*R. albus*, *R. flavefaciens* and *F. succinogenes*), fungi and protozoa and should be the reason of the increase in acetate molar percentage and ruminal NDF degradability with RF supplementation. The increase in fibrolytic microbes would cause the level of rumen acetate to increase[30]. Dietary fibre was degraded to acetate by bacteria, fungi and protozoa through the secretion of fibrolytic enzymes[31]. Fungi can degrade plant lignocellulosic tissues which are resistant to bacteria or protozoa, and approximately 10 % of the VFA production and 30 % of fibre digestion could be attributed to protozoa in the rumen[32]. In addition, RF supplementation increased the populations of *P. ruminicola* and *Rb. amylophilus*, and this also contributed to an increase in NDF digestibility. Fondevila & Dehority observed that digestion of cellulose increased when fibrolytic bacteria were co-cultured with amylolytic bacteria compared with fibrolytic species alone[33]. The responses of microbial population and cellulase activity observed suggested that dietary supplementation with RF was essential for rumen microbes. RF, in the form of flavin adenine dinucleotide and flavin mononucleotide, functions in an array of cellular electron transport processes including the metabolisms of carbohydrate, lipid and amino acids, cell signalling as well as protein folding, thereby playing a crucial role in cellular proliferation and growth[5,34]. Moreover, studies *in vitro* observed that RF addition promoted cellulose digestion and the growth of *R. albus* and *R. fiavefaciens*[10–12] and that addition of some B vitamins including RF stimulated the growth of protozoa[35].

Activities of α-amylase and protease were unaltered with RF addition and were in agreement with the unchanged *B. fibrisolvens* population. Rumen *B. fibrisolvens* is considered as one of the most important bacteria in the hydrolysis of starch and protein[36,37]. The results suggested that RF might not be an essential nutrient for *B. fibrisolvens*. Likewise, Gill & King observed that RF addition did not affect the growth of *B. fibrisolvens in vitro*[38]. Since *B. fibrisolvens* was ingested by protozoa more rapidly than other bacteria in the rumen[35], the change of *B. fibrisolvens* population should also be affected by the increase of protozoa population with RF supplementation. Therefore, the effect of RF addition on *B. fibrisolvens* needs to be verified by further study.

Rumen ammonia-N comes from feed protein degradation and the digestion of bacteria by protozoa[39]. In contrast to the unchanged CP degradability and increased protozoa population, ammonia-N concentration decreased with RF supplementation. The results indicated that more ammonia-N might be incorporated into microbial protein, as reflected by the higher urinary total purine derivatives excretion. The increment in rumen total VFA concentration suggested that more available energy and carbon source were provided to microbes to synthesise protein. Moreover, rumen ammonia-N content was sufficient to support the maximum microbial protein synthesis. The lower ammonia-N (8·33 mg/100 ml) was observed in bulls added medium RF and was above 5·0 mg/100 ml, a level considered to be suitable for microbial protein production[39]. The increase in total purine derivatives excretion represented an increase in the supply of microbial N to the intestine[40] and should be the cause for the increment of total tract CP digestibility with RF supplementation.

Conclusion

Dietary supplementation with RF had no influence on DM intake but tended to increase average daily gain in bulls. Supplementation with RF had positive impacts on nutrient digestion, rumen total VFA production, microbial growth and enzymatic activity in bulls. Dietary supplemented RF was mainly required for rumen fibrolytic microbial growth and feed fibre digestion and had the potential to stimulate microbial protein production. No further increase in performance and rumen total VFA concentration was observed when increasing addition level of RF from 600 to 900 mg/d in bulls.

Acknowledgements

The authors thank the staff of Shanxi Agriculture University dairy calves unit for care of the animals. All authors read and approved the manuscript.

This work was supported by a grant from Key Research and Development project of Shanxi Province (201903D211012 and 201903D221001) and Key Research and Development project of Jinzhong City of Shanxi Province (Y192009).

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

The authors declare that no conflicts of interest exist.

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