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Supplementing lactating dairy cows with a vitamin B_{12} precursor, 5,6-dimethylbenzimidazole, increases the apparent ruminal synthesis of vitamin B_{12}

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Cobalamin (CBL), the biologically active form of vitamin B_{12}, and its analogs, are produced by bacteria only if cobalt supply is adequate. The analogs differ generally by the nucleotide moiety of the molecule. In CBL, 5,6-dimethylbenzimidazole (5,6-DMB) is the base in the nucleotide moiety. The present study aimed to determine if a supplement of 5,6-DMB could increase utilization of dietary cobalt for synthesis of CBL and change ruminal fermentation, nutrient digestibility, omasal flow of nutrients and ruminal protozoa counts. Eight ruminally cannulated multiparous Holstein cows (mean ± standard deviation = 238 ± 21 days in milk and 736 ± 47 kg of BW) were used in a crossover design. Cows were randomly assigned to a daily supplement of a gelatin capsule containing 1.5 g of 5,6-DMB via the rumen cannula or no supplement. Each period lasted 29 days and consisted of 21 days for treatment adaptation and 8 days for data and samples collection. Five corrinoids, CBL and four cobamides were detected in the total mixed ration and the omasal digesta from both treatments. The dietary supplement of 5,6-DMB increased ($P = 0.02$) apparent ruminal synthesis of CBL from 14.6 to 19.6 (s.e.m. 0.8) mg/day but had no effect ($P > 0.1$) on apparent ruminal synthesis of the four analogs. The supplement of 5,6-DMB had no effect ($P > 0.1$) on milk production and composition, or on protozoal count, ruminal pH and concentrations of volatile fatty acids and ammonia nitrogen in rumen content. The supplement had also no effect ($P > 0.1$) on intake, omasal flow and apparent ruminal digestibility of dry matter, organic matter, NDF, ADF and nitrogenous fractions. Plasma concentration of CBL was not affected by treatments ($P = 0.98$). Providing a preformed part of the CBL molecule, that is, 5,6-DMB, increased by 34% the apparent ruminal synthesis of CBL by ruminal bacteria but had no effect on ruminal fermentation or protozoa count and it was not sufficient to increase plasma concentrations of the vitamin. Even though the efficiency of cobalt utilization for apparent synthesis of CBL was increased from 2.0% to 2.7% by the 5,6-DMB supplement, this improved efficiency was still very low. Further research is needed to identify the factors affecting efficiency of utilization of cobalt for synthesis of CBL by the bacterial populations in rumen.

Keywords: dairy cow, vitamin B_{12}, cobalt, 5,6-dimethylbenzimidazole, ruminal microflora

Implications

Unlike other B vitamins, vitamin B_{12} is not present in plants and is produced only by bacteria if cobalt supply is adequate. Therefore, dairy cows rely on synthesis of the vitamin by the bacteria present in rumen to cover their requirements. However, the proportion of cobalt used for these syntheses is low. Providing a preformed part of the vitamin B_{12} molecule (5,6-dimethylbenzimidazole) increased by 34% apparent synthesis of the vitamin by ruminal bacteria but failed to increase substantially efficiency of cobalt utilization. The results highlight the lack of knowledge on nutritional factors driving ruminal production of vitamin B_{12}.

Introduction

Increasing vitamin B_{12} supply of dairy cows with an adequate folate status has been reported to improve efficiency of energy metabolism in early lactation (Girard and Matte, 2005; Graulet *et al.*, 2007; Preynat *et al.*, 2009). However, as much as 80% of a dietary supplement of cyanocobalamin, the synthetic form of vitamin B_{12}, was catabolized in the rumen (Girard *et al.*, 2009b). Thus, the use of such supplements

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in commercial dairy farms is not economically viable. Unlike other B vitamins, vitamin B_{12} (cobalamin, CBL) is produced only by bacteria and archaebacteria if cobalt supply is adequate (Martens *et al.*, 2002). Even ciliate protozoa present in rumen need vitamin B_{12}, which they obtain by ingestion of these vitamin B_{12}-synthesizing bacteria (Bonhomme *et al.*, 1982). In addition, bacteria present in the rumen use dietary cobalt to produce molecules chemically related to CBL but devoid of biological activities for the host; these molecules are called vitamin B_{12} analogs and their presence in rumen content has been reported in previous studies (Ford *et al.*, 1953a; Dawbarn *et al.*, 1957; Gawthorne, 1970a; Dryden and Hartman, 1971).

The molecule of vitamin B_{12} is composed of four major parts: the corrin ring, the nucleotide moiety, the aminopropanol residue linking the nucleotide to the corrin ring and the B ligand linked to the cobalt atom within the corrin ring. In CBL, 5,6-dimethylbenzimidazole (5,6-DMB) is the base in the nucleotide moiety. In addition to CBL, six cobamides, molecules in which the 5,6-DMB in the nucleotide moiety is replaced by other bases and one cobinamide (COB), which lacks the nucleotide moiety have been detected in bovine ruminal content, duodenal and ileal digesta, and feces (Dryden and Hartman, 1971; Girard *et al.*, 2009a, 2009b). Increasing the proportion of forages in the diet has frequently been reported to increase CBL in rumen without effect on total vitamin B_{12} (CBL + analogs; Sutton and Elliot, 1972; Walker and Elliot, 1972; Santschi *et al.*, 2005). Moreover, apparent ruminal synthesis of CBL is negatively correlated with rumen pH (Schwab *et al.*, 2006), starch disappearance (Sutton and Elliot, 1972) or starch intake (Schwab *et al.*, 2006) and positively correlated with ADF or NDF intakes (Sutton and Elliot, 1972; Schwab *et al.*, 2006). Use of probiotics able to maintain a higher rumen pH during experimental induction of subacute ruminal acidosis also prevented the decrease in CBL concentration in rumen content observed at that time (Chiquette *et al.*, 2012). Seemingly in contradiction with the effects of rumen pH on CBL concentration in rumen, Cannizzo *et al.* (2012) observed that plasma concentrations of total vitamin B_{12} increased in cows with a rumen pH smaller than 5.6. However, this observation is in accordance with Walker and Elliot (1972) and Sutton and Elliot (1972) who reported that decreasing the proportion of forages in the diet increased serum concentrations of total vitamin B_{12} (Sutton and Elliot, 1972; Walker and Elliot, 1972) but decreased the proportion of CBL (Sutton and Elliot, 1972).

Nevertheless, it has been long recognized that the primary factor affecting the amounts of CBL and analogs synthesized by ruminal bacteria is cobalt (Gawthorne, 1970a). However, the proportion of dietary cobalt used for these synthetic processes is relatively low in sheep and cows, varying from 3% to 15% (Smith and Marston, 1970; Stemme *et al.*, 2008; Girard *et al.*, 2009b). *In vitro* studies demonstrated that addition of different bases to the culture media enhanced the synthesis of the corresponding cobamides at the expense of the other forms (Ford *et al.*, 1955; Gawthorne, 1970b). Rickard *et al.* (1975) observed that feeding 5,6-DMB increased production of 'true vitamin' B_{12} (CBL) in rumen of sheep but had no effect on apparent ruminal synthesis of total vitamin B_{12}. Therefore, the main objective of the present study was to determine if, in dairy cows, a daily supplement of 5,6-DMB could increase utilization of dietary cobalt for synthesis of CBL at the expense of vitamin B_{12} analogs. Effects of the 5,6-DMB supplement on animal performance, ruminal fermentation and protozoa counts, omasal flow of nutrients and nutrient digestibility in rumen were also studied.

Materials and methods

Care and handling of the animals were conducted as outlined in the guidelines of the Canadian Council on Animal Care (2009), and the study was approved by the Institutional Animal Care Committee of the Dairy and Swine Research and Development Centre, Sherbrooke, Québec, Canada.

Animals, experimental design and treatments

Eight ruminally cannulated multiparous Holstein cows averaging (mean ± s.d.) 238 ± 21 days in milk and 736 ± 47 kg of BW at the beginning of the experiment were randomly assigned to one of two treatments in a crossover design. Daily treatments were administered intraruminally via gelatin capsules containing or not 1.5 g of 5,6-dimethylbenzimidazole (Sigma-Aldrich, St Louis, MO, USA). Cows were fed twice daily at 0800 and 2000 h a total mixed ration (Table 1). Mineral and vitamin supply was calculated to meet or exceed the National Research Council (2001) recommendations and provided 0.1 mg Co/kg of DM. Orts were collected daily at 0700 h, and the amount of feed offered to the cows was adjusted daily to yield refusals equal to ~5% to 10% of intake. Cows were

Table 1 *Ingredient and nutrient composition of the TMR fed to Holstein cows supplemented or not with 5,6-DMB*

Dietary ingredients (% of diet DM)	
Corn silage	32.5
Grass silage	32.5
Cracked corn	23.6
Soybean meal	6.10
Canola meal	0.64
Corn distillers grain	0.96
Heated soybean meal	0.64
Corn gluten meal	0.96
Calcium carbonate	0.30
Sodium chloride	0.10
Mineral and vitamin premix	1.60
Nutrient composition	
DM (% of fresh matter)	42.2
CP (% of DM)	14.8
NDF (% of DM)	37.6
ADF (% of DM)	24.2
Ash (% of DM)	7.86
Cobalt (mg/kg of DM)	1.4

TMR = total mixed ration; 5,6-DMB = 5,6 dimethylbenzimidazole; DM = dry matter; CP = crude protein.

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housed in a tie stall barn and had free access to water throughout the experiment. Each period lasted 29 days (total of 58 days) and consisted of 21 days for treatment adaptation and 8 days for data and samples collection.

Feed sampling and analyses

Forage samples were collected twice a week and were immediately analyzed by near infrared reflectance spectrometry (Agri-Analyse Laboratoire Agricole, Sherbrooke, Québec, Canada), to adjust, when necessary, the amounts of energy and protein supplements in the total mixed ration to maintain as much as possible a consistent dietary nutrient composition throughout the experimental period. Samples of diet ingredients, total mixed ration and orts were collected in the last 3 days (days 27 to 29) of each period, dried at 55°C for 48 h, and ground to pass through a 1-mm screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA, USA) before analytical procedures. Samples were analyzed for analytical DM (method 930.15; Association of Official Analytical Chemists, 2006) and ash with a thermogravimetric analyzer (model TGA-601; Leco Corporation, St Joseph, MI, USA); total nitrogen (N) using micro-Kjeldahl analysis (Kjeltec 2400 instrument; Foss Analytical, Hillerød, Denmark; method 976.06; Association of Official Analytical Chemists, 2006); NDF and ADF with the Ankom^{200} fiber analyzer (Ankom Technology, Fairport, NY, USA) using heat-stable α-amylase and sodium sulfite (Van Soest *et al.*, 1991); and cobalt by atomic absorption spectrometry (Varian Vista AX-CCD, Varian Instruments, Mulgrave, Australia).

Animal performance, milk and plasma sampling and analyses

Cows were milked twice daily at ~0800 and 2000 h, and milk yield was recorded at each milking. Milk samples from a.m. and p.m. milkings were collected from day 27 (p.m.) to day 29 (a.m.) of each experimental period, preserved in tubes containing 2-bromo-2-nitropropane 1,3 diol, and kept at 4°C until shipped for determination of fat, protein, lactose and milk urea N by mid-IR reflectance spectroscopy (Valacta, Sainte-Anne-de-Bellevue, Québec, Canada). Concentrations and yields of milk components and milk urea N were computed as the weighted means from a.m. and p.m. milk yields on each test day.

On day 27, blood samples were collected by venipuncture of the coccygeal vein <1 h after the morning feeding using Vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing EDTA for vitamin B_{12} determinations. All tubes were immediately placed on ice and centrifuged at 4ºC for 15 min at 3300 × **g**. Plasma was kept frozen at − 20ºC until further analysis. Plasma concentrations of vitamin B_{12} were measured in duplicate by radioassay for two replicate samples (SimulTRAC-S Radioasssay kit, Vitamin B12 (^{57}Co)/Folate (^{125}I), MP Biomedicals, Diagnostics Division, Orangeburg, NY, USA).

Omasal sampling and analyses

Spot samples of omasal digesta leaving the rumen were collected through the reticulo-omasal orifice from the eight ruminally cannulated cows using the omasal sampling technique developed by Huhtanen *et al.* (1997) and Ahvenjärvi *et al.* (2000), as adapted by Reynal *et al.* (2003). The following omasal digesta markers were used: indigestible NDF (Huhtanen *et al.*, 1994) for the large particle phase (LP), $YbCl_3$ (modified from Siddons *et al.*, 1985) for the small particle phase (SP) and Cr-EDTA (Udén *et al.*, 1980) for the fluid phase (FP). A marker solution containing $YbCl_3$, Cr-EDTA and $^{15}NH_4SO_4$ with 10 atom percentage excess ^{15}N (Isotec, Miamisburg, OH, USA) as a bacterial marker was prepared as described by Reynal and Broderick (2005). A sample of 500 ml of omasal digesta (background) was taken from each cow before the beginning of markers infusion on day 22 of each period to determine the natural abundance of ^{15}N. Cows were then pulse-dosed with 3.0 l of the same markers solution used during the continuous infusion. The external markers Cr-EDTA, $YbCl_3$ and $^{15}NH_4SO_4$ were continuously infused into the rumen from days 23 to 28 (mean = 125 h of infusion) using four peristaltic pumps (Masterflex L/S model no. 7523 − 50, Cole-Parmer Instrument Co., Barrington, IL, USA) at an average constant rate of 3.02 kg/day providing daily 2.56 g of Cr, 2.34 g of Yb and 0.24 g of ^{15}N. Omasal sampling was initiated ~72 h after beginning the markers infusion with samples taken six times daily at 1-h intervals during 2 consecutive days in each period to represent a 12-h feeding cycle as follows: 0, 1, 2, 3, 4 and 5 h on day 27 and 6, 7, 8, 9, 10 and 11 h on day 28 with 0 h representing the time of the morning meal. The omasal sampling tube was kept inserted into the reticulo-omasal orifice for the entire collection of omasal digesta, which lasted ~7 h/day. Before each sampling point it was necessary to confirm the location of the sampling tube and occasionally it had to be repositioned into the omasal canal. It was also necessary a few times to unplug the holes in the end of the sampling tube because of the presence of coarse digesta. At each of the six daily sampling times, a 470-ml spot sample of omasal digesta was collected and split under continuous mechanical agitation into two subsamples of 70 and 400 ml. The six daily 70-ml subsamples were pooled and stored on ice inside a refrigerator (4°C) for the duration of the daily omasal digesta collection. These six daily 70-ml subsamples were pooled into a single composite of 420 ml per cow and transported to the laboratory for bacterial isolation. The six daily 400-ml subsamples were stored at −20°C and pooled over 2 days to obtain a single 4.8-l composite from each cow in each period for later separation into the three omasal digesta phases (LP, SP and FP).

The fluid-associated bacteria (FAB) and particle-associated bacteria (PAB) were isolated from the daily 420-ml composites from each cow on each of the 2 sampling days using filtration and differential centrifugation as described earlier in detail (Brito *et al.*, 2009). The resulting FAB and PAB pellets were stored at −20°C, lyophilized, ground with a mortar and pestle, and finally pooled by cow per period by mixing equal amounts of dry matter for later analysis. The 4.8-l pooled omasal composites were thawed at room temperature, separated into the three omasal phases (LP, SP and FP)

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as described by Brito *et al.* (2009), and stored at −20°C until lyophilized. After lyophilization, these samples were ground through a 1-mm screen before analyses of digesta markers.

Concentrations of Cr, Yb and indigestible NDF in LP and SP and Cr and Yb in FP were determined using the methods detailed earlier (Brito *et al.*, 2009). Marker concentrations were used to physically recombine DM from the lyophilized FP, SP and LP in the correct proportions to reconstitute the omasal true digesta (OTD) flowing out of the rumen using the triple-marker method of France and Siddons (1986). Concentrations of Cr, Yb and indigestible NDF were distinctly greater in, respectively, the FP, SP and LP, thus allowing for successful application of the triple marker method. On a dry matter basis, SP and LP subsamples were mixed in the correct proportions based on the digesta markers to yield a 2-g sample that was sequentially ground through a 1-mm screen and a 0.5-mm screen (Marathon Electric mill, Wausau, WI, USA) and defined as particle phase (PF).

Reconstituted OTD samples were analyzed for analytical DM, ash, total N, NDF and ADF as described previously for diet ingredients, total mixed rations and orts. Extracts from OTD samples were prepared and analyzed for ammonia-N (NH_3-N) as described by Brito *et al.* (2009).

Samples of FAB, PAB, PF and background omasal digesta were prepared for non-ammonia nitrogen (NAN) and ^{15}N analyses as described in detail elsewhere (Brito *et al.*, 2009). Both NAN and ^{15}N were analyzed with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK) at the Stable Isotope Facility of the University of California-Davis, USA. Bacterial samples (PAB and FAB) were also analyzed for analytical dry matter (overnight at 105°C) and ash (16 h at 550°C).

Ruminal sampling and analyses

Samples of whole ruminal contents (about 200 g) were taken from the ventral sac of the eight ruminally cannulated cows at 0 (pre-feeding), 1, 2, 3, 4, 5, 6, 8 and 10 h after the morning feeding on day 29 of each period. Ruminal digesta samples were strained through two layers of cheesecloth and pH was measured immediately (pH/temp meter 199 Model No. 3D, Fisher Scientific, Pittsburgh, PA, USA). Two 10-ml samples were then preserved by addition of 0.2 ml of 50% H_2SO_4 and stored at −20°C until analysis. Samples were thawed at room temperature, centrifuged (25 200 × **g**, 15 min, 4°C), and supernatants analyzed for NH_3-N as previously described and for volatile fatty acids (VFA) with a GLC equipment (Hewlett-Packard 6890N, Hewlett-Packard Inc., Montreal, QC, Canada) equipped with a flame-ionization detector and a 7683B model autosampler as described by Chiquette *et al.* (2008). Five hundred ml of ruminal fluid and 250 g of solid digesta were collected from each ruminally cannulated cow at 0 (pre-feeding), 3, 6 and 9 h after the morning feeding, blended and strained through two layers of cheesecloth. A 3-ml portion of the strained ruminal fluid was preserved using 3 ml of methyl green formalin-saline solution for protozoa enumeration (Ogimoto and Imai, 1981). Protozoa samples were stored at room temperature in the dark until counting. Protozoa were enumerated microscopically using a Levy-Hausser counting chamber (Hausser Scientific, Horsham, PA, USA). Each sample was counted twice, and if the average of the duplicates differed by more than 10%, the counting was repeated.

Analyses of vitamin B_{12} and analogs in feeds and OTD

Analyses of CBL and its analogs were performed on feeds and OTD samples. Sample preparation and analysis of CBL and its analogs by liquid chromatography–MS (LC–MS) were performed as described by Allen and Stabler (2008) using the following standards: CBL, the biologically active form in mammals; COB, a CBL without the base, ribose and phosphate groups or substitution of 5,6-DMB by adenine, ADE; benzimidazole, BZA; cresol, CRE; 2-CH_3-adenine, MADE; 5-CH_3-benzimidazole, MBZA; 5-CH_3O-benzimidazole, MOMZA; 5-CH_3O, 6-CH_3-benzimidazole, MOMBZA; 2-CH_3-S-adenine, MSADE; napthimidazole, NZA; 5-OH-benzimidazole, OHBZA and phenol, PHE. All data are presented as CN-CBL equivalents.

Both 'true' (CBL) and total (CBL + analogs) vitamin B_{12} were also measured by radioassays using either pig intrinsic factor (Sigma) or cow saliva as binding proteins as described by Santschi *et al.* (2005) except that 5 μl of NaCN 1 M per ml of solution was added during the extraction process. Intrinsic factor binds preferentially CBL, whereas haptocorrin present in saliva binds most corrinoids (Combs, 2012). Concentration of analogs was obtained by difference between total (haptocorrin) and true vitamin B_{12} (intrinsic factor).

Omasal flow calculations

Feed intake was measured throughout the duration of the experiment. It is important to note, however, that dry matter intake reported on Table 2 was calculated by averaging daily intakes from days 23 to 29 (i.e. total sampling week), whereas dry matter and other nutrient intakes reported on Tables 3 and 4 were calculated by averaging daily intakes during omasal sampling (days 26 to 28). Omasal flow of NAN was determined by difference between total N and NH_3-N flows. Total NAN flowing past the omasal canal was assumed to be composed by PAB NAN, FAB NAN and non-NH_3 non-bacterial N (NANBN). The natural abundance of ^{15}N in samples from background omasal digesta averaged 0.36086 (s.d. 0.002083) atom %. Enrichment of ^{15}N was defined as ^{15}N atom % excess above the natural abundance of ^{15}N measured in the background omasal digesta samples.

Assuming that FAB and PAB were representative of bacteria flowing with the FP and the PF, respectively, omasal flows of FAB NAN, PAB NAN, total bacterial NAN, NANBN and OM truly digested in the rumen (OMTDR) were calculated as follows:

$$\text{FAB NAN flow} = \text{FP NAN flow} \times (\text{FP } ^{15}\text{N APE} \div \text{FAB } ^{15}\text{N APE})$$

$$\text{PAB NAN flow} = \text{PF NAN flow} \times (\text{PF } ^{15}\text{N APE} \div \text{PAB } ^{15}\text{N APE})$$

$$\text{Total bacterial NAN flow} = \text{FAB NAN flow} + \text{PAB NAN flow}$$

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Table 2 *Dry matter intake, milk production and composition and ruminal variables in Holstein cows supplemented or not with 5,6-DMB*

	Treatments			
Item	Control	5,6-DMB	s.e.m.	*P*-value
Dry matter intake (kg/day)	23.0	23.2	0.56	0.55
Milk yield (kg/day)	25.5	25.2	1.02	0.49
Milk fat (g/kg)	37.7	37.6	1.73	0.80
Milk fat (kg/day)	0.95	0.92	0.052	0.20
Milk protein (g/kg)	35.3	35.5	0.69	0.51
Milk protein (kg/day)	0.88	0.87	0.031	0.50
Milk lactose (g/kg)	45.0	44.7	1.02	0.07
Milk lactose (kg/day)	1.13	1.10	0.45	0.16
Milk urea nitrogen (mg/dl)	9.44	9.36	0.578	0.80
Ruminal pH	6.22	6.24	0.04	0.49
Ruminal ammonia-nitrogen (mg/dl)	10.6	9.9	1.23	0.57
Ruminal volatile fatty acids (mM)	106	108	4.7	0.52
Ruminal acetate (A; mM)	69.9	71.6	2.66	0.48
Ruminal propionate (P; mM)	20.6	21.0	1.56	0.59
Ruminal butyrate (mM)	11.5	11.1	0.78	0.89
Ruminal A : P	3.5	3.5	0.15	0.99

5,6-DMB = 5,6-dimethylbenzimidazole.

Omasal flows of NANBN and RUP, RDP supply and OM TDR were calculated as follows:

$$\text{NANBN flow} = \text{total NAN flow} - \text{total bacterial NAN flow}$$

$$\text{RUP flow} = \text{total CP flow} - (\text{total microbial NAN flow} \times 6.25)$$

$$\text{RDP supply} = \text{total CP intake} - \text{RUP flow}$$

$$\text{FAB or PAB DM flow} = \text{FAB or PAB NAN flow} \div (\,\% \text{ FAB or PAB NAN} \div 100)$$

$$\text{FAB or PAB OM flow} = (\text{FAB or PAB DM flow} \times \,\% \text{ FAB or PAB OM}) \div 100$$

$$\text{Total bacterial OM flow} = \text{FAB OM flow} + \text{PAB OM flow}$$

$$\text{OMTDR} = \text{OM intake} - (\text{omasal OM flow} - \text{total bacterial OM flow})$$

where flows and intakes are in grams per day or kilograms per day and NAN concentrations are in grams per gram of OM.

Statistical analyses

Data were analyzed using the MIXED procedure of SAS (Version 9.3; SAS Institute Inc., Cary, NC, USA) according to a crossover design. The following model was fitted for all variables with no repeated measures over time:

$$Y_{ijk} = \mu + S_i + C_j(S)_i + T_k + ST_{ik} + E_{ijk}$$

where: Y_{ijk} is the dependent variable; μ the overall mean; S_i the mean effect of the i^{th} crossover sequence group, $C_j(S)_i$ the mean effect of j^{th} cow nested within i^{th} sequence, T_k the mean effect of the l^{th} treatment, ST_{ik} the interaction between i^{th} crossover sequence group and k^{th} treatment (same as period effect) and E_{ijk} the random residual variation. All terms were considered fixed except $C_j(S)_i$ and E_{ijk} that were considered random.

The following model was fitted for variables with repeated measures over time (ruminal pH, ruminal concentrations of NH_3-N and VFA, and ruminal protozoal count):

$$Y_{ijkl} = \mu + S_i + C_j(S)_i + T_k + ST_{ik} + E1_{ijk} + H_l + TH_{kl} + E2_{ijkl}$$

where Y_{ijkl} is the dependent variable, μ the overall mean, S_i the mean effect of the i^{th} crossover sequence group, $C_j(S)_i$ the mean effect of j^{th} cow nested within i^{th} sequence, T_k the mean effect of k^{th} treatment, ST_{ik} the interaction between i^{th} crossover sequence group and k^{th} treatment (same as period effect), $E1_{ijk}$ the whole-plot random residual variation, H_l the mean effect of l^{th} hour postfeeding analyzed as repeated measures, TH_{kl} the interaction between k^{th} treatment and l^{th} hour postfeeding and $E2_{ijkl}$ the subplot random residual variation. The spatial covariance structure with the lowest Akaike information criterion was retained in the final model. The subject of the repeated measurements was defined as cow (period). All terms were considered fixed, except $C_j(S)_i$, $E1_{ijk}$ and $E2_{ijkl}$, which were considered random.

All reported values are least square means and standard error of the mean. Differences were considered significant at $P \leqslant 0.05$ and trends were declared at $0.05 < P \leqslant 0.10$.

Results

Five corrinoids, CBL and four cobamides, ADE, MADE, MSADE and OHBZA were detected in the total mixed ration and the omasal digesta (Table 3). The supplement of 5,6-DMB increased ($P = 0.02$) the apparent ruminal synthesis of CBL by 34% but had no effect ($P > 0.1$) on apparent ruminal synthesis of the four analogs, OHBZA, MADE, ADE and MSADE (Table 3). When analyzed by radioassays using intrinsic factor and cow saliva (haptocorin) as binding proteins, the supplement of 5,6-DMB had no effect ($P > 0.1$) on apparent ruminal synthesis of total vitamin B_{12} and analogs but increased ($P = 0.01$) synthesis of 'true vitamin B_{12}' by 38% (Table 3). The correlation between CBL measured directly using the LC–MS method or as 'true vitamin B_{12}' by radioassay using intrinsic factor as binding protein was high ($r = 0.97$, $P \leqslant 0.0001$). Total corrinoids (summation of the forms quantified using the LC–MS method) and total vitamin B_{12} measured by radioassay using cow saliva (haptocorrin as binding protein) were also correlated ($r = 0.67$; $P = 0.004$) but the correlation between the two methods to estimate the analogs (summation of the forms other than CBL measured by the LC–MS method or difference between the two radioassays) was not significant ($r = 0.40$; $P = 0.13$).

Plasma concentrations of vitamin B_{12} ($P = 0.98$) were not affected by treatments, averaging 201 and 202 (s.e.m. 10) pg/ml for control and treated cows, respectively.

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Table 3 *Intake, omasal flow and apparent ruminal synthesis of corrinoids in Holstein cows supplemented or not with 5,6-DMB*

	Treatments			
Item	Control	5,6-DMB	s.e.m.	*P*-value
Corrinoids (measured by LC–MS)[1]				
OHBZA				
Intake (mg/day)	0.008	0.008	0.0003	0.97
Omasal flow (mg/day)	10.1	9.6	0.48	0.32
Ruminal apparent synthesis (mg/day)	10.1	9.6	0.48	0.32
MADE				
Intake (mg/day)	0.043	0.043	0.0015	0.93
Omasal flow (mg/day)	48.5	46.3	1.71	0.23
Ruminal apparent synthesis (mg/day)	48.4	46.2	1.71	0.23
ADE				
Intake (mg/day)	0.056	0.056	0.0019	0.74
Omasal flow (mg/day)	7.6	7.7	0.89	0.97
Ruminal apparent synthesis (mg/day)	7.6	7.6	0.89	0.97
MSADE				
Intake (mg/day)	0.002	0.002	0.0001	0.69
Omasal flow (mg/day)	1.9	1.8	0.12	0.75
Ruminal apparent synthesis (mg/day)	1.9	1.8	0.12	0.81
Total analogs				
Intake (mg/day)	0.11	0.11	0.004	0.86
Omasal flow (mg/day)	68.1	65.4	2.09	0.28
Ruminal apparent synthesis (mg/day)	68.0	65.3	2.09	0.28
CBL				
Intake (mg/day)	0.14	0.14	0.005	0.70
Omasal flow (mg/day)	14.8	19.8	0.85	0.02
Ruminal apparent synthesis (mg/day)	14.6	19.6	0.84	0.02
Corrinoids (measured by radioassays)[2]				
True vitamin B_{12}				
Intake (mg/day)	0.79	0.80	0.028	0.95
Omasal flow (mg/day)	20.5	27.9	1.56	0.01
Ruminal apparent synthesis (mg/day)	19.7	27.1	1.55	0.01
Vitamin B_{12} analogs				
Intake (mg/day)	0[3]	0		
Omasal flow (mg/day)	53.6	54.4	4.49	0.91
Ruminal apparent synthesis (mg/day)	53.6	54.4	4.49	0.91
Total vitamin B_{12}				
Intake (mg/day)	0.28	0.28	0.009	0.62
Omasal flow (mg/day)	74.1	82.2	5.51	0.34
Ruminal apparent synthesis (mg/day)	73.8	82.0	5.51	0.34

5,6-DMB = 5,6-dimethylbenzimidazole; LC–MS = liquid chromatography–MS.
[1]Corrinoids measured by LC–MS: cobamides with substitution of 5,6-DMB by 5-OH-benzimidazole, OHBZA; 2-CH_3-adenine, MADE; adenine, ADE; 2-CH_3-S-adenine, MSADE; summation of these forms = total analogs; cobalamin, CBL. All data are presented as CN-Cbl equivalents.
[2]Corrinoids measured by radioassays using intrinsic factor (true vitamin B_{12}) or haptocorrin from cow saliva (total vitamin B_{12}). Vitamin B_{12} analogs were obtained by differences between total and true vitamin B_{12}.
[3]In total mixed ration, total vitamin B_{12} determined with a radioassay using haptocorrin as binding protein was smaller than true vitamin B_{12} measured with a radioassay using intrinsic factor as binding protein.

A daily dose of 5,6-DMB had no effect ($P > 0.1$) on DMI, milk production and composition (Table 2). Ruminal pH and concentrations of VFA and NH_3-N in ruminal content were also unaffected ($P > 0.1$; Table 2) by the 5,6-DMB supplement. Similarly, protozoal count did not differ ($P = 0.98$) between treatments; averaging 585 631 and 584 312 (s.e.m. 44 745) protozoa/ml of ruminal fluid, for the control and treated groups, respectively. The supplement had also no effect ($P \geqslant 0.2$) on intakes, omasal flows as well as apparent ruminal digestibility of DM, OM, NDF, ADF and nitrogenous fractions (Table 4). Although omasal flow of total bacterial NAN (i.e. FAB NAN + PAB NAN flows) did not differ ($P = 0.96$) between treatments, feeding 5,6-DMB tended ($P = 0.08$) to increase (+10%) FAB NAN flow and decreased (−9%; $P = 0.02$) PAB NAN flow.

Discussion

Cyanocobalamin, pseudovitamin B_{12} (ADE), factor A (MADE), factor B (COB) and factor C have been detected in silage,

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Table 4 *Intake, omasal flow and ruminal digestibility of DM, OM, NDF, ADF and nitrogenous fractions in Holstein cows supplemented or not with 5,6-DMB*

	Treatments			
Item	Control	5,6-DMB	s.e.m.	*P*-value
DM				
Intake (kg/day)	22.3	22.5	0.76	0.80
Flow (kg/day)	12.5	13.0	0.48	0.26
Apparent digestibility in rumen (% of intake)	43.9	42.1	1.06	0.75
OM				
Intake (kg/day)	20.6	20.7	0.70	0.79
Flow (kg/day)	9.9	10.1	0.36	0.31
Apparent digestibility in rumen (% of intake)	52.1	51.0	0.69	0.20
True digestibility in rumen (% of intake)	70.5	69.0	0.80	0.23
NDF				
Intake (kg/day)	8.4	8.4	0.29	0.77
Flow (kg/day)	3.2	3.3	0.16	0.23
Apparent digestibility in rumen (% of intake)	62.1	60.4	1.04	0.31
ADF				
Intake (kg/day)	5.4	5.4	0.18	0.74
Flow (kg/day)	2.0	2.0	0.09	0.31
Apparent digestibility in rumen (% of intake)	63.6	62.7	0.81	0.38
Nitrogen				
Intake (g/day)	533.6	536.9	17.25	0.82
Flow (g/day)	512.2	521.4	13.78	0.52
Apparent digestibility in rumen (% of intake)	3.9	3.0	1.70	0.55
True digestibility in rumen (% of intake)	62.4	60.5	1.42	0.36
Ammonia nitrogen flow (g/day)	17.6	17.8	1.43	0.95
NAN flow (g/day)	494.6	503.7	13.92	0.52
NANBN flow (g/day)	183.0	192.6	5.23	0.24
FAB NAN flow (g/day)	145.4	160.1	6.10	0.08
PAB NAN flow (g/day)	166.2	150.9	8.12	0.02
Total bacterial NAN flow (g/day)	311.6	311.1	11.70	0.96
RDP supply (g/day)	2081	2041	101	0.70
RUP flow (g/day)	1254	1315	34.4	0.26
g of bacterial NAN/kg of OMADR	29.2	29.6	0.80	0.55
g of bacterial NAN/kg of OMTDR	21.5	21.8	0.50	0.45

DM = dry matter; OM = organic matter; 5,6-DMB = 5,6-dimethylbenzimidazole; NAN = non-ammonia nitrogen; NANBN = non-ammonia non-bacterial nitrogen; FAB = fluid-associated bacteria; PAB = particle-associated bacteria; OMADR = organic matter apparently digested in the rumen; OMTDR = organic matter truly digested in the rumen.

although the plant species was not mentioned (Ford *et al.*, 1953b). In the present study, CBL and four cobamides, ADE, MADE, MSADE and OHBZA were quantified in the total mixed ration, whereas only CBL and COB were detected in total mixed rations used by Girard *et al.* (2009a and 2009b), even if those rations contained some ingredients similar to those used in the present study, such hay, grass silage or corn silage. Nevertheless, dietary concentrations of corrinoids (CBL and analogs; 11 μg/kg of DM) and CBL (6 μg/kg of DM) were similar to values observed by Girard *et al.* (2009a and 2009b). The total amount of corrinoids in the total mixed ration (0.25 mg/day) represented only 0.3% of the omasal flow of total corrinoids. Vitamin B_{12} is not synthesized by plants; therefore, the small amounts detected in feedstuffs are likely due to bacterial contamination from soil or during silage fermentation process (Martens *et al.*, 2002; Combs, 2012). In the present study, CBL and four cobamides, ADE, MADE, MSADE and OHBZA, were quantified in omasal digesta, whereas COB and two supplementary cobamides were also detected in duodenal digesta of dairy cows fed different diets (Girard *et al.*, 2009a and 2009b).

Radioassays using intrinsic factor from pig stomach are available commercially and allow for rapid determination of 'true vitamin B_{12}' in a large number of samples. Both intrinsic factor and haptocorrin have a high affinity for CBL but haptocorrin has a greater affinity for analogs than intrinsic factor (Kolhouse and Allen, 1977; Adjalla *et al.*, 1993). Measurements of CBL in feed and omasal digesta by LC–MS or by a radioassay using intrinsic factor as binding protein were highly correlated. However, quantification of analogs by difference between the two radioassays was less accurate than by the LC–MS method, possibly because the affinity of haptocorrin for corrinoids varies among the different cobamides.

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As observed *in vitro* (Ford *et al.*, 1955; Gawthorne, 1970b) and *in vivo* (Rickard *et al.*, 1975), in studies done with sheep, supplementing 5,6-DMB to dairy cows increased apparent synthesis of CBL in rumen by 34% (5 mg/day) but had no effect on the amounts of analogs produced. The authors of both studies concurred that, when cobalt supply is adequate, 5,6-DMB availability may become limiting (Gawthorne, 1970b; Rickard *et al.*, 1975), although the metabolic pathway for anaerobic synthesis of 5,6-DMB by ruminal bacteria is not fully elucidated (Renz *et al.*, 1993; Roth *et al.*, 1996). On a weight basis, cobalt represents 4.4% of the CBL molecule, thus, in the present study 0.64 and 0.86 mg of Co were incorporated into CBL for control and 5,6-DMB supplemented diets, respectively. With a basal diet providing 1.4 mg of Co/kg of DM, efficiency of cobalt utilization for CBL synthesis represented 2.0% and 2.7% for control and 5,6-DMB supplemented diets, respectively. Efficiency of cobalt utilization in the present study was lower than expected. Indeed, in sheep, efficiency of cobalt utilization decreased as the dietary concentration of cobalt increased (Smith and Marston, 1970). In cows fed different diets, efficiency of cobalt utilization for CBL synthesis in the rumen has been reported to be 7.1%, 9.5% and 4.4% for diets providing 0.17, 0.29 and 2.5 mg of Co/kg of DM, respectively (Stemme *et al.*, 2008; Girard *et al.*, 2009a). Therefore, it is likely that the efficiency of bacterial utilization of cobalt for CBL synthesis is influenced by the ingredients and chemical compositions of diets and their effects on microflora and fermentation in rumen.

Already in 1953, Ford *et al.* (1953a) reported that the relative proportions of vitamin B_{12} and its analogs produced in rumen depend not only on the composition of the microflora but also on the nature of the diet. Only four species out of the 21 studied species of microorganisms present in rumen, *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Butyrivibrio fibrisolvens* and an unnamed species, were able to synthesize corrinoids with the first two species producing the greatest amounts of vitamin B_{12} and analogs (Dryden *et al.*, 1962). Moreover, changes in culture media, such as energy sources or precursors of the nucleotide base, affect the proportions of CBL and analogs (Dryden *et al.*, 1962). Many bacterial species producing analogs (cobamides) in pure culture preferentially use 5,6-DMB when it is available in the culture medium (Hazra *et al.*, 2013). These authors reported to have detected free 5,6-DMB in microbial communities and hypothesized that bacterial species not able to synthesize 5,6-DMB could produce CBL if the nucleotide base is available in their environment. In the present study, there appears to be a shift in bacterial population occurring with the addition of 5,6-DMB, which favored the growth of FAB, this fraction comprising the known vitamin B_{12}-synthesizing bacteria, at the expense of PAB. Nevertheless, this shift in the order of 10% for both bacterial NAN flows was not sufficient to impact on rumen fermentation parameters or protozoa counts.

In conclusion, despite the significant increase in the apparent ruminal synthesis of CBL in response to intraruminal supplementation of 5,6-DMB, this improvement was not large enough to enhance markedly the efficiency of cobalt utilization for CBL synthesis. The current results highlight the lack of knowledge on the nutritional factors driving ruminal production of vitamin B_{12} in presence of a sufficient cobalt supply as well as on the interactions among bacterial populations for preferential production of CBL over analogs.

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