Effects of dietary nitrogen levels and carbohydrate sources on apparent ruminal synthesis of some B vitamins in dairy cows

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ABSTRACT

Effects of nitrogen level and carbohydrate source on apparent ruminal synthesis (ARS) of thiamin, riboflavin, niacin, vitamin B₆, folates, and vitamin B₁₂ were evaluated using 4 lactating Holstein cows distributed in a 4 × 4 Latin square design with treatments following a 2 × 2 factorial arrangement. Cows were fitted with cannulas in the rumen and proximal duodenum. The treatments were 2 N levels and 2 carbohydrate sources. The diet with the high N level provided 14% crude protein, calculated to meet 110% of the protein requirements and an adequate supply in rumen-degradable protein, whereas the diet with the low N level contained 11% crude protein, calculated to meet 80% of the protein requirements with a shortage in rumen-degradable protein. Carbohydrate source treatments differed by their nature (i.e., high in starch from barley, corn, and wheat, or high in fiber from soybean hulls and dehydrated beet pulp). All 4 diets were isoenergetic, based on corn silage, and had the same forage-to-concentrate ratio (60:40, dry matter basis). Duodenal flow was determined using YbCl₃ as a marker. Each B-vitamin ARS was calculated as duodenal flow minus daily intake. The intake of several B vitamins varied among treatments, but because the animals consumed a similar amount of feed every day (average of 20 kg of dry matter/d) the difference was mostly due to vitamin content of each ingredient and their relative proportion in the diets. Decreasing N concentration in the diet reduced vitamin B₆ duodenal flow and increased its apparent ruminal degradation. It also decreased duodenal flow and ARS of folates. The high-starch diets increased duodenal flow and ruminal balance of riboflavin, vitamin B₆, and folates, whereas the high-fiber diets increased vitamin B₁₂ ARS and duodenal flow. These effects on apparent synthesis are possibly due to changes in ruminal fermentation.

Key words: dairy cow, B-vitamin ruminal synthesis, nitrogen, carbohydrate source

INTRODUCTION

In the early 20th century, several studies demonstrated synthesis of B vitamins in the rumen of dairy cows. From these data, it was concluded that ruminants with a balanced diet and a functional rumen could meet their requirements in B vitamins (Bechdel and Honeywell, 1927; Bechdel et al., 1928; Kon and Porter, 1954), and therefore deficiencies in these nutrients should not be a concern for dairy nutritionists. Even though they are required in small amounts, B vitamins play an essential role as they act as cofactors or coenzymes in the metabolism of carbohydrates, lipids, proteins, and nucleic acids (Combs, 2012). Several researchers have shown that B-vitamin supplements can improve milk yield, composition, and metabolic efficiency in high-producing dairy cows (Jaster and Ward, 1990; Girard and Matte, 1998; Shaver and Bal, 2000; Graulet et al., 2007). However, the response to these dietary supplements is highly variable and one of the major reasons is because the amounts of vitamins provided by the basal diet and the synthesis in the rumen are unknown. Dietary characteristics, such as carbohydrate source and protein supply, affect fermentation and microorganisms in the rumen (Johnson and Johnson, 1995; Hristov et al., 2001; Belanche et al., 2012). For example, easily fermentable carbohydrates, such as starch, are more efficient than other carbohydrates, such as cellulose, to promote microbial growth (Bach et al., 2005), whereas reduction in nitrogen supply can lead to a lower rumen microbial diversity and abundance (Belanche et al., 2012). To our knowledge, information on the effect of N level and carbohydrate source on B-vitamin apparent ruminal synthesis (ARS) is very limited. Therefore, our study was undertaken to identify the effects of these 2 parameters and their interaction on intake, duodenal flow and ARS of some B vitamins in lactating dairy cows.

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MATERIALS AND METHODS

The experimental procedures were conducted in accordance with national legislation issued by the French government (Ministère de l'Alimentation, de l'Agriculture et de la Pêche, 2009) and international recommendation (Canadian Council on Animal Care, 1993) on the care and use of laboratory animals.

Animals and Treatments

The experimental procedures were described in details by Fanchone et al. (2013). Briefly, we used 4 Holstein cows fitted with cannulas in the rumen and the proximal duodenum and weighing on average 662 ± 62 kg at 71 ± 10 d of lactation (mean $\pm$ SD) at the beginning of the experiment. The experimental design was a 4×4 Latin square design with each experimental period lasting 28 d, with 22 d of adaptation to the diet and 6 d for sampling. Treatments were 2 levels of N and 2 carbohydrate sources in a factorial arrangement. The high level of N met 110% of protein requirements of cows whereas the low level covered 80% of these requirements, expressed in the French system of protein digestible in the intestine (INRA, 2007) with a shortage in rumen-degradable protein (NRC, 2001). The difference between the 2 carbohydrate sources was based on the nature of concentrates (i.e., rich in starch or rich in fiber). Diets were corn silage-based, had the same forage-to-concentrate ratio (60:40, DM basis), were isoenergetic, and formulated to meet net energy theoretical requirements (INRA, 2007; Table 1). The amount of feed offered daily was restricted at 95% of voluntary intake measured at the beginning of the study and adjusted at the beginning of each experimental period to net energy theoretical requirements (Fanchone et al., 2013). Each ingredient was weighed individually before distribution as a TMR. Intake was the difference between the amounts of feed offered and refused daily for each cow from d 22 to 28. Water was provided ad libitum.

Sample and Data Collection

Measurements and sampling procedures to characterize ruminal fermentation (VFA, pH, $\text{NH}_3\text{-N}$), NAN duodenal flow, rumen digestibility, and N balance have been described by Fanchone et al. (2013).

Laboratory Analyses

B-vitamin concentrations were determined in feed and duodenal digesta samples. Concentrations of thia-

min, riboflavin, niacin, and vitamin B_6 were determined by HPLC (Varian Prostar, Lake Forest, CA) with a solvent delivery system (model 210), an autosampler (model 410), and a fluorescence detection system (model 363). Foliates were analyzed with a microbiological microtiter plate test (VitaFast Folic Acid, R-Biopharm Inc., Marshall, MI) and vitamin B_{12} was analyzed by radio-assay (SimulTRAC-S Vitamin B_{12} [Co^{57}]/Folate [I^{125}], MP Biomedicals, Solon, OH). Cobalt concentration in feed was determined by atomic absorption spectrometer (PerkinElmer, Waltham, MA).

Thiamin (B1). Thiamin concentrations were analyzed in duplicate by HPLC according to a method adapted from Böttcher and Böttcher (1986). Standard curves were prepared as followed: a standard stock solution was prepared by dissolving 33.7 mg of thiamin hydrochloride (Sigma-Aldrich, Oakville, ON, Canada) in a final volume of 100 mL of 0.01 M H_2SO_4 solution (Fisher Scientific, Ottawa, ON, Canada). Then, 400 μL of the solution was diluted in 1,600 μL of ultrapure water to obtain an initial concentration of 200 μM thiamin, which was used to obtain a 7-level standard curve (0.3–7.5 μM). For the feed and digesta, 0.1 g of sample and 5 mL of H_2SO_4 were mixed in a 15-mL polypropylene conical tube and autoclaved (100°C, 15 min). After cooling in an ice bath, pH was adjusted to 4.5 with 4.0 M $\text{C}_2\text{H}_3\text{NaO}_2$ buffer (Sigma-Aldrich) and 500 μL of 1% reduced glutathione (Sigma-Aldrich) was added to each tube. Next, according to the methods adapted from Hyun and Tamura (2005) and Jakobsen (2008), 1 mL of an aqueous trienzyme mix was added, containing papain (60 U, Sigma-Aldrich), α -amylase (10,167 U, Sigma-Aldrich), and acid phosphatase (14 U, Sigma-Aldrich). The samples were incubated (60 min) in an ultrasonic bath (Ultrasonic Cleaners, 40 kHz, Cole-Parmer, Montréal, QC, Canada) and then autoclaved to inactivate the enzymes (100°C, 5 min). After cooling in an ice bath, the volume was completed to 7 mL with ultrapure water and centrifuged (900 $\times g$, 10 min, 4°C). Immediately following centrifugation, the supernatant (1 mL) was mixed with 225 μL of oxidative solution [175 μL of 50% NaOH and 50 μL of 5% K_3FeCN_6 (Avantor Performance Materials, Center Valley, PA) per sample]. After incubation (5 min), approximately 0.25 g of NaCl was added to each tube. Following mixing, 2 mL of 2-butanol was added, the samples were shaken for 1 min and then 10 μL was injected into the HPLC. The analysis was carried out on a Polaris-NH₂ column (250 mm $\times$ 4.6 mm, 5 μm ; Agilent, Mississauga, ON, Canada) preceded by a guard column (MetaGuard Polaris-NH₂, 4.6 mm, 3 μm , Agilent). The mobile phase was composed of 250 mL of 0.04 M KH_2PO_4 buffer and 750 mL of acetonitrile at a flow rate of 1.5 mL/min, with the fluorimetric detection

system adjusted at 425 and 370 nm for emission and excitation, respectively. Mean intra-assay coefficients of variation were 2.4 and 2.8% for feed and digesta, respectively, and recovery rate was 89%.

Riboflavin (B_2). Riboflavin concentrations were determined in duplicate according to a method adapted from Giguère et al. (2002). Initial solutions of riboflavin (2,656 μM) and flavin-adenine dinucleotide (**FAD**; 2,411 μM ; Sigma-Aldrich) were used to obtain standard curves with 6 concentration levels (5.31 to 79.70 and 5.30 to 79.57, respectively). All forms of the vitamin were transformed into riboflavin for analysis and the FAD curve was made to confirm the efficiency of the

reaction. Feed and digesta samples (0.2 g) were acidified in 10 mL of 0.1 M HCl (Fisher Scientific) in 50-mL polypropylene conical tubes and autoclaved for 30 min at 100°C. After cooling in an ice bath, pH was adjusted to 4.5 with 4.0 M $C_2H_3NaO_2$ buffer (Sigma-Aldrich) and 500 μL of 1% reduced glutathione (Sigma-Aldrich) were added in each tube. Next, 1 mL of a trienzyme solution as used for thiamin analysis was added. The samples were incubated (1 h 25 min) in an ultrasonic bath (Ultrasonic Cleaners, 40 kHz, Cole-Parmer) and then autoclaved (100°C, 5 min). After cooling in an ice bath, the volume was made up to 15 mL with ultrapure water and centrifuged ($900 \times g$, 10 min, 4°C).

Table 1. Ingredient, chemical composition, nutritive value, and vitamin concentrations of the diets fed to dairy cows receiving high-starch or high-fiber concentrate with a low or high N level¹

Item	Low N		High N	
	Starch	Fiber	Starch	Fiber
Ingredient, ² % of DM				
Corn silage	40.5	40.5	40.5	40.5
Hay	10.0	10.0	10.0	10.0
Dehydrated alfalfa	9.0	9.0	9.0	9.0
Molasses chopped wheat straw	6.3	0	5.2	0
Cereal-based concentrate ³	30.6	0	24.3	0
Soybean hulls	0	31.0	0	22.4
Dehydrated beet pulp	0	9.0	0	9.0
Soybean meal	3.6	0	10.8	8.6
Urea	0	0.5	0.2	0.5
Chemical composition, % of DM				
OM	94.3	93.3	93.8	93.0
NDF	36.2	50.7	36.1	47.1
ADF	18.8	30.7	18.4	27.4
CP	11.0	11.1	14.2	14.4
Starch	32.4	15.1	29.0	15.3
Nutritive value				
NE _L , kJ/kg of DM	6,683	6,535	6,753.0	6,650
PDIE, ⁴ g/kg of DM	87	87	99	100
PDIN, ⁵ g/kg of DM	71	70	96	97
RDP, ⁶ g/kg of DM	74	76	98	97
RUP, ⁶ g/kg of DM	36	39	46	50
MP, ⁶ g/kg of DM	88	84	97	96
Total cobalt, mg/kg of DM	0.695	1.050	0.948	1.135
Vitamin concentration, mg/kg of DM				
Thiamin	2.63	1.59	2.45	1.61
Riboflavin	58.90	51.30	58.38	52.23
Niacin	106.12	107.71	106.56	107.72
Vitamin B ₆	10.24	7.87	9.95	8.06
Folates	0.48	0.67	0.61	0.76
Vitamin B ₁₂	0.004	0.004	0.004	0.004

¹Ingredients, chemical compositions, and nutritive values from Fanchone et al. (2013).

²Each cow received 200 g/d of a vitamin and trace element premix provided in the daily diet: 2.5% P, 20% Ca, 4.5% Mg, 3.5% Na, 400,000 IU/kg of vitamin A, 120,000 IU/kg of vitamin D₃, 1,600 mg/kg of vitamin E, 1,300 mg/kg of copper sulfate, 5,000 mg/kg of zinc oxide, 3,500 mg/kg of manganese oxide, 90 mg/kg of calcium iodate, 36 mg/kg of cobalt carbonate, and 20 mg/kg of sodium selenite; Galaphos Midi Duo, CCPA, Aurillac, France.

³Cereal-based concentrate: 39% barley, 46% wheat, and 15% corn, on a DM basis.

⁴PDIE = protein digested in the small intestine supplied by rumen-undegraded dietary protein and by microbial protein from rumen-fermented OM (INRA, 2007).

⁵PDIN = protein digested in the small intestine supplied by rumen-undegraded dietary protein and by microbial protein from rumen degraded N (INRA, 2007).

⁶Calculated from NRC feed tables (NRC, 2001) using the actual DMI level (on average, 3.04% BW).

The supernatant (500 μL) was combined with 200 μL of 15% HClO_4 solution, and samples were then boiled for 10 min. Following centrifugation ($900 \times g$, 10 min, 4°C), the supernatant (400 μL) was mixed with 200 μL of 4.0 M $\text{C}_2\text{H}_3\text{NaO}_2$ and 25 μL of 2% acid phosphatase solution [20 mg of phosphatase/mL of $\text{C}_2\text{H}_7\text{NO}_2$ buffer (50 mM, pH 4.0)]. Samples were incubated (37°C , 18 h) in a water bath to convert FAD into riboflavin. Following centrifugation ($10,000 \times g$, 10 min, 4°C), the supernatants were injected into the HPLC. Analysis was carried out with a Pursuit 5 μm C18 column (150 $\times$ 4.6 mm; Varian, Mississauga, ON, Canada) preceded by a guard column (MetaGuard Pursuit 5 μm C18, 4.6 mm; Agilent). The mobile phase was composed of 80 mL of $\text{C}_2\text{H}_7\text{NO}_2$ buffer (pH 4.0), 800 mL of ultrapure water, and 200 mL of acetonitrile, at a flow rate of 1.0 mL/min and the fluorimetric detection system adjusted at 520 and 450 nm for emission and excitation, respectively. The mean intra-assay coefficients of variation were 4.3 and 2.3% for feed and digesta, respectively, and recovery rate was 102%.

Niacin (B3). Concentrations of the 2 forms of niacin, nicotinic acid (NA) and nicotinamide (NAM) were analyzed in duplicate by HPLC with fluorometric detection based on adaptation of methods of Mawatari et al. (1991), Lahély et al. (1999), and Ndaw et al. (2002). Standard solutions were prepared by dissolving 0.01 g of NA and NAM (Sigma-Aldrich) in 100 mL of ultrapure water. Six concentrations of NA (5.04 to 99.99 μM) and NAM (5.16 to 100.00 μM) were used for the standard curves. Then, 0.2 g of sample, feed, or digesta was acidified in 10 mL of 0.1 M HCl (Fisher Scientific) in a 50-mL polypropylene conical tube and autoclaved 30 min at 100°C . After cooling in an ice bath, pH was adjusted to 4.5 with 4.0 M $\text{C}_2\text{H}_3\text{NaO}_2$ buffer (Sigma-Aldrich) and 500 μL of 1% reduced glutathione (Sigma-Aldrich) were added in each sample. Next, the same trienzyme mix as for thiamin analyses was applied. The samples were incubated (1 h 25 min) in an ultrasonic bath (Ultrasonic Cleaners, 40 kHz, Cole-Parmer) and then autoclaved (100°C , 5 min). After cooling in an ice bath, the volume was adjusted to 15 mL with ultrapure water and centrifuged ($900 \times g$, 10 min, 4°C). The supernatants (2 mL) were collected and 20 μL were injected into the HPLC. Samples were run through a Pursuit 5 μm C18 column (150 $\times$ 4.6 mm; Agilent) preceded by a guard column (MetaGuard Pursuit 5 μm C18, 4.6 mm; Agilent). The postcolumn photochemical reaction was carried out in a polytetra-fluoroethylene tube (10 m $\times$ 0.5 mm), which was wound around a black light (Black-Ray, model XX-20BLB, 300–400 nm with a filter excluding the 254-nm line, 115 V, 60 Hz, 60 A; UVP, Upland, CA). The mobile phase consisted of 0.07 M KH_2PO_4 buffer containing 0.075 M H_2O_2 and 5.1 μM

copper II sulfate (Sigma-Aldrich). The flow rate was adjusted to 1.5 mL/min, and the fluorimetric detector operated at an excitation wavelength of 322 nm and an emission wavelength of 380 nm. Total niacin daily intake, duodenal flow, and ARS were the summation of NA and NAM for these variables. The mean intra-assay coefficients of variation were 10.56 and 4.4% in feed and digesta, respectively, and recovery rate was 96%.

Vitamin B₆. The 3 forms of vitamin B₆, pyridoxamine (PAM), pyridoxal (PAL), and pyridoxine (PYR), were determined in duplicate by adaptation of methods described by Srivastava and Beutler (1973) and Matte et al. (1997). Four stock solutions made in ultrapure water (500 $\mu\text{g}/\text{mL}$; Sigma-Aldrich) of PAM, PAL, PYR, and pyridoxal-5'-phosphate (P5P) were diluted (one-tenth for PAM, PAL, and P5P; one-thirtieth for PYR) to obtain standard solutions (respectively, 207, 246, 202, and 81 μM). Feed and digesta samples were analyzed for PAM, PAL, and PYR simultaneously, whereas P5P was converted to PAL before analysis. A separate standard curve was made for P5P to validate the efficiency of the transformation into PAL. In a 50-mL polypropylene conical tube, 10 mL of 0.1 M H_2SO_4 solution was added to 0.2 g of sample as solid material and autoclaved (100°C , 15 min). After cooling in an ice bath, pH was adjusted to 4.5 with 4.0 M $\text{C}_2\text{H}_3\text{NaO}_2$ buffer (Sigma-Aldrich) and 500 μL of 1% reduced glutathione (Sigma-Aldrich) was added to each tube. Next, adapted from the method of Hyun and Tamura (2005), 1 mL of an aqueous solution of papain (60 U, Sigma-Aldrich) and α -amylase (10,167 U, Sigma-Aldrich) was added. The samples were incubated (1 h 25 min) in an ultrasonic bath (Ultrasonic Cleaners, 40 kHz, Cole-Parmer) and then autoclaved (100°C , 5 min). After cooling in an ice bath, the volume was made up to 20 mL with ultrapure water and centrifuged ($900 \times g$, 10 min, 4°C). Immediately following centrifugation, the supernatant (1 mL) was incubated (37°C , 18 h) with 25 μL of 2% phosphatase acid solution [20 mg of phosphatase per mL of $\text{C}_2\text{H}_7\text{NO}_2$ buffer (50 mM, pH 4.0)] to convert P5P into PAL. The samples were then centrifuged ($10,000 \times g$, 10 min, 4°C) before being injected into the HPLC, using the same model of column as for niacin analyses. The mobile phase for vitamin B₆ consisted of 0.09 M H_2SO_4 at a flow rate of 1.5 mL/min. Emission and excitation wavelengths of the fluorimetric detection system were respectively 290 and 395 nm. Total vitamin B₆ daily intake, duodenal flow, and ARS were the summation of PAM, PAL, and PYR for these variables. Although concentrations of some forms of the vitamin were lower than the first standard, the mean intra-assay coefficient of variation were 4.5 and 3.4% in feed and digesta, respectively, and recovery rate was 95%.

Folates (B_9). The method of extraction was adapted from AOAC International (2005; 960.46) using the following extraction solution: 14.2 g of Na_2HPO_4 (Fisher Scientific) and 10 g of $\text{C}_6\text{H}_8\text{O}_7$ (Sigma-Aldrich) in 1 L of ultrapure water (pH 7.3). In a 50-mL polypropylene conical tube, 5 mL of this phosphate buffer was mixed with 0.1 g of solid material. According to the method adapted from Hyun and Tamura (2005), 100 μL of a papain solution made in ultrapure water (60 U, Sigma-Aldrich) was added and all samples were incubated (37°C , 1 h 30 min). The samples were autoclaved to inactivate the enzyme (100°C , 5 min) and cooled in an ice bath, and then 100 μL of α -amylase (10167 U, Sigma-Aldrich) and 200 μL of conjugase solutions made in ultrapure water (5 mg/mL, Pel-Freez Biologicals, Rogers, AR) were added. All samples were incubated (37°C , 3 h) before autoclaving (100°C , 5 min). Following cooling at room temperature, the volume was completed to 40 mL with ultrapure water. Next, the tubes were centrifuged ($5,000 \times g$, 10 min, 4°C) and the supernatants were used to determine folate concentration using the commercial microbiological microtiter plate test (VitaFast Folic Acid, R-Biopharm Inc.). This procedure used *Lactobacillus rhamnosus*, which responds to the widest variety of folate biological active forms (Bird and McGlohon, 1972; Tamura et al., 1972). Samples were analyzed in triplicate on the microtiter plate and the mean intra-assay coefficient of variation were 4.4 and 3.5% for feed and digesta, respectively, and recovery rate was 100%.

Vitamin B_{12} . The method of extraction was adapted from AOAC International (2005; 952.20) using the following extractive solution: 13 g of Na_2HPO_4 (Fisher Scientific), 12 g of $\text{C}_6\text{H}_8\text{O}_7$ (Sigma-Aldrich), and 10 g of $\text{Na}_2\text{S}_2\text{O}_5$ (Fisher Scientific) in 1 L of ultrapure water. A sample of 0.1 g of solid material was hydrolyzed with 20 mL of extractive solution and 150 μL of 1.0 M NaCN (Sigma-Aldrich) were added before autoclaving (100°C , 10 min). Subsequently, the tubes were cooled at room temperature and the pH was adjusted with 3.3 M NaOH at pH 6.2 to 6.5. The final volume was 30 mL after addition of ultrapure water and samples were centrifuged ($3,000 \times g$, 10 min, 4°C). A volume of 200 μL of the supernatant was used to determine the concentration of the biologically active form of vitamin B_{12} using a commercial kit (SimulTRAC-S Vitamin B_{12} [Co^{57}]/Folate [I^{125}], MP Biomedicals). All samples were analyzed in duplicate and the mean intra-assay coefficient of variation in digesta was 3.4%, whereas that of feed is not presented because many samples were below detection limit. Recovery rate was 99%.

Cobalt. Cobalt concentration in the dietary ingredients was determined by atomic absorption spectrometer with flame stoichiometric air-acetylene at 240.7

nm based on an adaptation of the method of Bellanger (1988). Aliquots of 1.0 g of feed were dry ashed for 18 h at 500°C with a hold at 350°C for 1 h. Then, dry ashed samples were dissolved in 6.0 M HCl and homogenized in an ultrasonic bath (Ultrasonic Cleaners, 40 kHz, Cole-Parmer) for 30 min. If needed, further dilutions were made with 1.0 M HCl. A coefficient of variation below 10% was accepted.

Calculations and Statistical Analyses

B-vitamin concentrations in the diets were calculated based on B-vitamin concentrations in each ingredient multiplied by the proportion of this ingredient in the experimental diets on a DM basis. Daily intake of each vitamin was calculated as the concentration of each vitamin in the diet multiplied by the amount ingested, on a DM basis. Calculation of DM duodenal flows was described by Fanchone et al. (2013). Duodenal flow of vitamins was calculated as B-vitamin concentrations in the duodenal sample, on a DM basis, multiplied by the daily DM flowing through the duodenum. The ARS was calculated as the duodenal flow minus daily intake.

Daily intake, duodenal flow, and ARS for each vitamin were analyzed according to a 4×4 Latin square design using the MIXED procedure of SAS (2008) according to the following model:

$$Y_{ijkl} = \mu + \beta_i + K_j + N_k + C_l + N_k \times C_l + \varepsilon_{ijkl}$$

where Y_{ijkl} is the studied variable, μ the overall mean, β_i the cow effect, K_j the period effect, N_k the N level effect, C_l the carbohydrate source effect, $N_k \times C_l$ their interaction effect, and ε_{ijkl} the residual error. The statistical model included N level, carbohydrate source, and their interaction as fixed effects and cow as a random effect. A factorial design was used to test the effect of the 2 levels of N, the 2 carbohydrate sources and their interaction. Differences were declared significant at $P < 0.05$ and as a tendency at $0.05 \leq P \leq 0.10$. When the interaction was or tended to be significant, a slice test was made to interpret the interaction. Pearson correlation coefficients were determined between B-vitamin ARS and dietary parameters.

RESULTS AND DISCUSSION

B-vitamin concentrations in diets and dietary ingredients are presented in Tables 1 and 2, respectively. Daily DMI (20 ± 0.5 kg/d; Fanchone et al., 2013) and duodenal flow of DM (12.4 ± 0.72 kg/d) were not affected by treatments ($P \geq 0.36$ for N level, carbohydrate source and their interaction). Therefore, differences in daily intake of B vitamins depended mostly on

Table 2. B-vitamin concentrations (mg/kg of DM $\pm$ SD; n = 4) in the dietary ingredients fed to the dairy cows

Item	Corn silage	Hay	Dehydrated alfalfa	Molasses wheat straw	Cereal-based concentrate ¹	Soybean hulls	Dehydrated beet pulp	Soybean meal
Thiamin	1.37 $\pm$ 0.03	1.49 $\pm$ 0.08	2.92 $\pm$ 0.09	1.45 $\pm$ 0.32	4.92 $\pm$ 0.24	1.73 $\pm$ 0.12	0.95 $\pm$ 0.03	1.96 $\pm$ 0.30
Riboflavin	73.2 $\pm$ 4.05	35.0 $\pm$ 2.96	100.4 $\pm$ 11.12	96.9 $\pm$ 20.08	30.6 $\pm$ 3.16	23.6 $\pm$ 4.12	20.2 $\pm$ 1.53	34.4 $\pm$ 6.81
Niacin	204.2 $\pm$ 4.26	15.6 $\pm$ 2.10	70.2 $\pm$ 11.65	46.6 $\pm$ 15.73	36.0 $\pm$ 0.98	44.6 $\pm$ 8.11	36.9 $\pm$ 2.54	44.7 $\pm$ 4.40
Vitamin B ₆	15.0 $\pm$ 1.88	4.2 $\pm$ 0.01	8.1 $\pm$ 0.59	15.9 $\pm$ 2.64	6.1 $\pm$ 0.07	1.6 $\pm$ 0.54	1.6 $\pm$ 0.23	3.8 $\pm$ 0.50
Folates	0.10 $\pm$ 0.02	0.38 $\pm$ 0.06	2.81 $\pm$ 0.16	0.21 $\pm$ 0.03	0.23 $\pm$ 0.02	1.13 $\pm$ 0.08	0.05 $\pm$ 0.01	1.96 $\pm$ 0.75
Vitamin B ₁₂	0.005 $\pm$ 0.002	0.011 $\pm$ 0.005	0.006 $\pm$ 0.002	0.003 $\pm$ 0.002	0.001 $\pm$ 0.001	0.001 $\pm$ 0.001	0.001 $\pm$ 0.001	0.001 $\pm$ 0.001

¹Cereal-based concentrate: 39% barley, 46% wheat, and 15% corn on a DM basis.

the vitamin concentrations of the ingredients and their relative proportions in the experimental diets, whereas differences in B-vitamin duodenal flow were mostly due to B-vitamin concentrations in digesta. Average vitamin intake, duodenal flow, and ARS are presented in Table 3.

Thiamin

Thiamin daily intake, duodenal flow, and ARS were not affected ($P \geq 0.28$) by the N level. Thiamin intake was greater with the high-starch diets ($P < 0.01$; 51.1 vs. 34.2 $\pm$ 2.36 mg/d; Table 3) as a consequence of the higher concentrations of thiamin in diets rich in starch (2.54 mg/kg of DM; Table 1) as compared with high-fiber diets (1.60 mg/kg of DM; Table 1). Nevertheless, thiamin duodenal flow or ARS did not differ statistically ($P \geq 0.41$) between carbohydrate sources. This is in accordance with the study of Schwab et al.

(2006), who found no effect of different dietary forage and nonfiber carbohydrate contents on thiamin ARS. Thiamin ARS was positively correlated only with total VFA, as previously observed by Schwab et al. (2006).

Riboflavin

Riboflavin intake did not differ ($P = 0.90$) between dietary N levels, but the high-N diets increased duodenal flow ($P = 0.03$; 1,156 vs. 1,481 $\pm$ 85.5 mg/d) and ARS ($P = 0.04$; 43 vs. 372 $\pm$ 95.2 mg/d) of riboflavin. These results are in accordance with the study of Lardin et al. (1944), who reported that the apparent synthesis of riboflavin was greater when the diet contained more degradable N (7.6 vs. 13.6 μ g of riboflavin/g of DM of rumen content). Feeding diets rich in starch resulted in greater intake and duodenal flow of riboflavin ($P = 0.01$; 1,172 vs. 1,050 $\pm$ 25.8 mg/d and $P = 0.01$; 1,545 vs. 1,091 $\pm$ 92.4 mg/d, respectively) and

Table 3. Intake, duodenal flow, and apparent ruminal synthesis (ARS) of thiamin, riboflavin, niacin, vitamin B₆, folates, and vitamin B₁₂ of cows receiving high-starch or high-fiber concentrate at a low or high N level

Item	Low N		High N		SEM	P-value		
	Starch	Fiber	Starch	Fiber		N	C ¹	N × C
Intake, mg/d								
Thiamin	52.6	31.7	49.5	36.7	3.34	0.76	<0.01	0.27
Riboflavin	1,193	1,033	1,151	1,067	36.5	0.90	0.01	0.35
Niacin	2,143	2,170	2,135	2,215	70.9	0.78	0.50	0.73
Vitamin B ₆	206	158	198	164	5.6	0.87	<0.01	0.23
Folates	9.9	14.0	11.6	15.9	0.52	0.01	<0.01	0.86
Vitamin B ₁₂	0.10	0.10	0.10	0.08	0.003	0.43	0.56	0.84
Duodenal flow, mg/d								
Thiamin	36.1	24.7	45.4	37.5	10.29	0.28	0.41	0.88
Riboflavin	1,362	949	1,729	1,233	130.6	0.03	0.01	0.78
Niacin	1,473	1,166	1,604	1,530	147.8	0.10	0.26	0.48
Vitamin B ₆	53.0	41.6	84.4	49.7	6.98	0.01	0.01	0.15
Folates	27.2	23.4	45.1	35.1	5.22	0.01	0.25	0.59
Vitamin B ₁₂	2.78	9.19	3.47	9.44	1.034	0.64	<0.01	0.85
ARS, mg/d								
Thiamin	-16.5	-7.0	-4.1	0.8	11.19	0.36	0.56	0.85
Riboflavin	169	-84	578	166	145.4	0.04	0.06	0.62
Niacin	-670	-1,003	-530	-684	138.7	0.11	0.14	0.56
Vitamin B ₆	-153	-116	-113	-115	9.6	0.04	0.11	0.09
Folates	17.3	9.4	33.5	19.2	5.14	0.02	0.07	0.58
Vitamin B ₁₂	2.70	9.12	3.38	9.36	1.033	0.65	<0.01	0.85

¹C = carbohydrates.

tended to increase ARS ($P = 0.06$; 373 vs. 41 ± 102.8 mg/d). This trend to increase ARS is in line with the study of Santschi et al. (2005b), in which a low-forage diet led to a greater riboflavin concentration in the solid-associated bacteria fraction than a high-forage diet. Hunt et al. (1943) showed that rumen riboflavin concentration increased when a rapidly fermentable carbohydrate source was added to the diet. In the present study, the diets were isoenergetic, but it can be hypothesized that the bacterial population responsible for starch degradation produces more riboflavin than the bacterial population involved in fiber degradation. This hypothesis is supported by the negative correlation between riboflavin ARS and NDF and ADF intakes, as well as with NDF ruminally digested (kg/d; Table 4). As recently performed for the human gut microbiota (Magnúsdóttir et al., 2015), a genome assessment of the B-vitamin biosynthetic pathway among rumen microbes would help to validate this hypothesis

and to understand the effect of the microbiota composition (in line with the diet composition) on B-vitamin synthesis. Riboflavin ARS was strongly negatively correlated with ruminal protein balance and had a high positive correlation with microbial synthesis efficiency, whether in the liquid-associated bacteria fraction or in the mixed bacteria fraction, which contained both solid- and liquid-associated bacteria. Lastly, ARS was positively correlated with NAN duodenal flows (Table 4). All these results would suggest, notwithstanding possible changes in ruminal bacteria species, that riboflavin synthesis in rumen was greater under dietary conditions promoting microbial growth (i.e., readily available sources of degradable carbohydrates and N).

Niacin

Daily intake of niacin was not affected by dietary treatments ($P \geq 0.50$). However, high-N diets tended

Table 4. Pearson correlation coefficients between B-vitamin apparent ruminal synthesis (ARS, mg/d) and dietary and digestive parameters

Item	B-vitamin ARS					
	Thiamin	Riboflavin	Niacin	Vitamin B ₆	Folates	Vitamin B ₁₂
Intake, kg/d						
DM	-0.31	-0.42	-0.15	-0.27	-0.40	0.29
OM	-0.44	-0.45	-0.18	-0.33	-0.38	0.22
NDF	0.09	-0.70**	-0.56*	0.15	-0.66**	0.85***
ADF	0.11	-0.70**	-0.58*	0.17	-0.67**	0.85***
Starch	-0.31	0.49	0.47	-0.39	0.45	-0.85***
N	0.15	0.23	0.28	0.31	0.31	0.22
Intake of vitamins, ¹ mg/d	-0.40	-0.15	-0.27	-0.64**	-0.36	-0.28
Ruminally digested, kg/d						
NDF	0.04	-0.61*	-0.49	0.18	-0.49	0.77***
Starch	-0.34	0.47	0.46	-0.43	0.47	-0.88***
Ruminal measures						
Ruminal protein balance, ² g of CP/kg of DMI	-0.20	-0.70**	-0.66**	-0.09	-0.39	0.29
VFA						
Total, mM	0.65**	0.17	0.45	0.31	-0.14	0.54*
Acetate, % total VFA	0.40	0.16	0.05	0.19	-0.01	0.46
Propionate, % total VFA	-0.17	-0.46	-0.42	-0.19	-0.25	0.08
Butyrate, % total VFA	-0.37	-0.02	0.14	-0.22	0.03	-0.50*
pH	0.07	-0.11	-0.38	0.19	-0.005	0.12
NH ₃ -N	0.32	-0.10	-0.10	0.45	-0.01	0.60*
LAB MSE, ³ g of N/kg of OMTRD	0.21	0.73**	0.54*	0.33	0.61*	-0.26
MB MSE, ⁴ g of N/kg of OMTRD	0.22	0.71**	0.57*	0.36	0.57*	-0.21
<i>Isotrichidae</i> /mL						
Time postfeeding: 0 h	-0.23	-0.12	-0.02	0.26	-0.00	0.19
Time postfeeding: 2.5 h	0.10	-0.34	-0.31	0.19	-0.33	0.43
<i>Ophryoscolecidae</i> /mL						
Time postfeeding: 0 h	0.18	0.05	0.33	0.34	-0.11	0.27
Time postfeeding: 2.5 h	0.35	0.23	0.35	0.35	0.10	0.34
Duodenal measures						
NAN duodenal flow, g/d	0.25	0.67**	0.68**	0.29	0.51**	-0.05

¹Intake of the corresponding B vitamin.

²Ruminal protein balance = (N intake - duodenal NAN flow) $\times$ 6.25/DMI.

³Liquid associated bacteria microbial synthesis efficiency, grams of microbial N/kilograms of OM truly ruminally digested (OMTRD; calculated using LAB as microbial reference sample).

⁴Mixed bacteria microbial synthesis efficiency, grams of microbial N/kilograms of OMTRD (calculated using MB as microbial reference sample).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

to increase niacin duodenal flow ($P = 0.10$; $1,320$ vs. $1,567 \pm 96.8$ mg/d), possibly due to a numerically decreased degradation of the vitamin in the rumen ($P = 0.11$; -837 vs. -608 ± 90.8 mg/d), regardless of carbohydrate source ($P \geq 0.14$; Table 3). In the present trial, niacin was degraded in the rumen (negative values for ARS) in all dietary treatments, contrary to other previous experiments where niacin ARS was positive (Miller et al., 1986; Santschi et al., 2005a; Schwab et al., 2006). However, niacin intake in this trial was up to 4 fold higher than those studies, which could explain the ruminal synthesis differences. Santschi et al. (2005a) also observed that increasing niacin intake by using a supplement had no effect on niacin duodenal flow because the supplementary niacin was almost completely destroyed in rumen. As with riboflavin, niacin ARS was negatively correlated with ADF and NDF intake. We found a strong negative correlation between ARS and ruminal protein balance and a positive correlation between ARS and microbial synthesis efficiency (LAB and MB) and with NAN duodenal flows (Table 4). These observations suggest that apparent degradation of niacin in rumen was reduced under the same conditions that promoted riboflavin ARS.

Vitamin B₆

The dietary N level had no effect on intake of vitamin B₆ ($P = 0.87$; Table 3), but the low-N diets decreased its duodenal flow ($P = 0.01$; 47.3 vs. 67.1 ± 4.57 mg/d). Vitamin B₆ intake ($P < 0.01$; 202 vs. 161 ± 3.9 mg/d) and duodenal flow ($P = 0.01$; 68.7 vs. 45.7 ± 4.94 mg/d) were greater with the starch diets. The carbohydrate source had no effect on apparent degradation (negative ARS) of vitamin B₆ in rumen of cows fed the high-N diets ($P = 0.93$), but starch increased the vitamin degradation in rumen of cows fed low-N diets ($P = 0.03$; interaction N level $\times$ carbohydrate source, $P = 0.09$; Table 3). Santschi et al. (2005a) also observed apparent ruminal degradation of vitamin B₆ in rumen, whereas Schwab et al. (2006) observed positive ARS. It is worthy of note that vitamin B₆ intake was 2.4 times greater in the study of Santschi et al. (2005a) than in Schwab et al. (2006). In the present study, vitamin B₆ ARS was negatively correlated with the intake of the vitamin, suggesting a possible downregulation of its synthesis by its availability (Table 4). This result is similar to what was observed by Castagnino et al. (2015).

Folates

Regardless of the diet composition, folate ARS was positive and values obtained in the current experiment

were in the same range as the ones reported in earlier studies (Santschi et al., 2005a; Schwab et al., 2006). The low-N diets decreased intake, duodenal flow, and ARS of folates ($P = 0.01$; 12.0 vs. 13.7 ± 0.34 mg/d; $P = 0.01$; 25.3 vs. 40.1 ± 3.41 mg/d; and $P = 0.02$; 13.3 vs. 26.4 ± 3.37 mg/d, respectively), although Lardinois et al. (1944) observed no direct correlation between increased urea intake and folate concentration in rumen content. The high-starch diets resulted in lower intakes of folates ($P < 0.01$; 10.7 vs. 15.0 ± 0.37 mg/d), had no effects on duodenal flow ($P = 0.25$), but tended to increase ARS ($P = 0.07$; 14.3 vs. 25.4 ± 3.64 mg/d). Increasing concentration of nonfiber carbohydrates has been reported to increase folate duodenal flow and ARS, whereas increasing forage proportion in the diet had opposite effects (Schwab et al., 2006).

Correlations between folate ARS and dietary parameters follow the same trends as riboflavin and niacin ARS. Folate ARS was strongly negatively correlated with ADF and NDF intakes and positively correlated with the microbial synthesis efficiency (LAB and MB) as well as with NAN duodenal flows (Table 4).

Vitamin B₁₂

The intake of vitamin B₁₂ was not affected by the N level or the carbohydrate source of the diet. In fact, intake of the vitamin was negligible (Tables 2 and 3) because vitamin B₁₂ is synthesized only by bacteria and, except for contamination, is therefore absent in feeds from plant origin (Combs, 2012). Consequently, as the supply via feed is very limited, values for duodenal flow and ARS were similar. The dietary level of N had no effects on vitamin B₁₂ ARS ($P = 0.65$; Table 3). Unlike all other B vitamins studied, fiber-rich diets enhanced ARS of vitamin B₁₂ ($P < 0.01$; 9.23 vs. 3.04 ± 0.730 mg/d, respectively). In accordance with Schwab et al. (2006), vitamin B₁₂ ARS had a strong positive correlation with ADF and NDF intakes, whereas a negative correlation was observed with starch intake. Similarly, the synthesis of vitamin B₁₂ was correlated positively and negatively with NDF and starch ruminally digested, respectively. Vitamin B₁₂ synthesis was positively correlated with total VFA, acetate, and ammonia-N, but negatively correlated with butyrate. Dryden and Hartman (1971) also observed a negative correlation between butyrate and vitamin B₁₂ concentration.

Cobalt is essential to produce vitamin B₁₂ (McDowell, 2000). In the present study, supplemental Co was similar among treatments (7.2 mg/d), but total Co intake was affected by the N level ($P < 0.01$; 21.1 vs. 17.6 ± 0.41 mg/d for high and low N levels, respectively) and the carbohydrate source ($P < 0.01$; 22.2 vs. 16.5 ± 0.44 mg/d for high-fiber and high-starch diets,

respectively). Therefore, we can hypothesize that the higher Co concentration in the fiber-rich diets induced a greater vitamin B₁₂ synthesis. These results are in accordance with the ones reported by Sutton and Elliot (1972), in which Co intake decreased as the concentrate content of the diet increased. They also observed a significantly lower vitamin B₁₂ synthesis with a forage-to-concentrate ratio of 40:60 compared with ratios of 70:30 and 100:0. Furthermore, in the current study, we observed a highly positive correlation between total Co intake and vitamin B₁₂ ARS ($P < 0.01$; $r = 0.79$). Vitamin B₁₂ ARS in the present experiment was much lower than the values observed by Schwab et al. (2006; 60.1–102.2 mg/d) or Santschi et al. (2005a; 73 mg/d) with diets with a higher total Co concentration than in the present study. Hedrich et al. (1973) also observed, in sheep, that increasing Co intakes resulted in a higher vitamin B₁₂ production. Consequently, it is difficult to dissociate the effects of Co supply itself on vitamin B₁₂ production in the rumen from those due to dietary carbohydrate source.

CONCLUSIONS

Overall, in the present study there was an apparent synthesis of riboflavin, folates, and vitamin B₁₂ but an apparent degradation of thiamin, niacin, and vitamin B₆ in rumen. The high-starch diets increased ARS of riboflavin and folates and reduced apparent ruminal degradation of vitamin B₆, whereas vitamin B₁₂ ARS was greater with the high-fiber diets. The low-N diets decreased ARS of riboflavin and folates. Overall, riboflavin, niacin, and folate apparent synthesis in rumen was negatively correlated with NDF and ADF intakes, whereas vitamin B₁₂ followed the opposite pattern. In the range of the studied diets, which all had the same forage-to-concentrate ratio, thiamin and vitamin B₆ were not influenced by the intake of nutrients. Moreover, only vitamin B₆ ARS was correlated with its intake. The current study confirmed that characteristics of the diet influence B-vitamin supply for dairy cows, probably by affecting microbial population and activity.

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