Effects of forage family on apparent ruminal synthesis of B vitamins in lactating dairy cows

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ABSTRACT

Effects of forage family (legume vs. grass) on apparent ruminal synthesis (ARS) and postruminal supply of B vitamins were evaluated in 2 experiments. Diets containing either alfalfa (AL) or orchardgrass (OG) silages as the sole forage were offered to ruminally and duodenally cannulated lactating Holstein cows in cross-over design experiments. Experiment 1 compared diets containing AL and OG [~23% forage neutral detergent fiber (NDF) and ~27% total NDF] offered to 8 cows in two 15-d treatment periods. Experiment 2 compared diets containing AL and OG (~25% forage NDF and ~30% total NDF) offered to 13 cows in two 18-d treatment periods. Thiamin, riboflavin, niacin, vitamin B₆, folates, and vitamin B₁₂ were analyzed in feeds and duodenal digesta. Apparent ruminal synthesis was calculated as the duodenal flow of each vitamin minus its intake. Forage family affected B vitamin intakes, duodenal flow, and ARS. In both experiments, AL diets increased vitamin B₆ and decreased folate intakes. In experiment 1, riboflavin and niacin intakes were greater with the OG diet, whereas in experiment 2 thiamin intake was greater but riboflavin intake was smaller with the OG diet. In spite of the low contribution of either silage to the dietary folate content, folate intake was greater with OG diets than AL due to the difference in soybean meal contribution between diets. Niacin and folate ARS were not affected by the forage family. Duodenal microbial nitrogen flow was positively correlated with ARS of riboflavin, niacin, vitamin B₆, folates, and vitamin B₁₂, but tended to be negatively correlated with thiamin ARS. Apparent ruminal synthesis of fo-

lates and vitamin B₁₂ appear to be related to microbial biomass activity. Changes in nutrient composition of the diets likely affected the microbial population in the rumen and their B vitamin metabolism.

Key words: apparent ruminal synthesis, B vitamin, dairy cow, forage family

INTRODUCTION

Studies on B vitamin requirements of cattle, conducted more than 60 yr ago, concluded that mature ruminants with a functional rumen did not require B vitamin supplements because the amounts of vitamins provided by the diet or synthesized by rumen microorganisms were sufficient to avoid apparition of deficiency symptoms (Bechdel et al., 1926; Kon and Porter, 1954). However, deficiency symptoms are the last stage of the deficiency; deficiency appears as soon as the supply is inferior to the needs, leading to biochemical changes (Combs, 2012). Reports of positive production responses to B vitamin supplementation in high-producing dairy cows (Girard and Matte, 1998, 2005; Schwab et al., 2005; Chen et al., 2011) suggest that, under some conditions, the need for B vitamins frequently exceed the supply from the diet and the synthesis by rumen microbes, leading to suboptimal milk production and metabolic efficiency. Shaver and Bal (2000) reported that supplementing dietary thiamin increased milk and component yields. A meta-analysis showed that a niacin supplement increased milk fat and protein yields and tended to increase feed efficiency (Schwab et al., 2005). Supplementary folic acid increased milk production of cows in the second lactation or greater (Girard and Matte, 1998). Girard and Matte (2005) observed that, when compared with a supplement of folic acid alone, a combined supplement of folic acid and vitamin B₁₂ increased milk and milk component yields in primiparous cows during early lactation. However, some studies observed no effect of vitamin supplements (Rosendo et al., 2004; Girard et al., 2005).

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A better understanding of dietary factors driving the fate of B vitamins in rumen of dairy cows is essential to define their B vitamin requirements. A limited number of studies have looked at the effects of dietary factors, such as forage-to-concentrate ratio or grain processing, on production of B vitamins by the ruminal microflora (Conrad and Hibbs, 1954; Hayes et al., 1966; Breves et al., 1981; Abel et al., 2001), but to our knowledge, no data are available on the effects of forage family. The objective of the present study was to describe the effect of forage family (legume or grass) on B-vitamin supply and apparent ruminal synthesis (**ARS**) in lactating dairy cows.

MATERIALS AND METHODS

Experimental procedures of the 2 studies were conducted on cows from the Michigan State University Dairy Cattle Teaching and Research Center, and approved by the Institutional Animal Care and Use Committee at Michigan State University (East Lansing, MI). Prior to calving, cows were cannulated ruminally (Bar Diamond Inc., Parma, ID) and duodenally with a gutter-type T cannula placed approximately 10 cm distal to the pylorus (Joy et al., 1997). Surgery was performed at the Department of Large Animal Clinical Science, College of Veterinary Medicine, Michigan State University. Experimental procedures, measurements, and analyses were described in detail by Voelker Linton and Allen (2008; experiment 1), and Kammes and Allen (2012; experiment 2).

Experiment 1

Cows and Treatments. Eight multiparous cows were assigned randomly to treatment sequence in a crossover design with a 14-d preliminary period followed by two 15-d treatment periods. The 2 treatments were diets containing either alfalfa (**AL**; *Medicago sativa* L.) silage, harvested at early bud stage, or orchardgrass (**OG**; *Dactylis glomerata* L.) silage, harvested at early boot stage as sole forage (Table 1). As both diets were formulated to contain 27% NDF and 23% forage NDF (Voelker Linton and Allen, 2008), the forage-to-concentrate ratios were 53:47 and 48:52 for AL and OG, respectively. Cows were fed ad libitum a TMR served once daily (1100 h) at 110% of expected intake.

Sample and Data Collection. Individual feed intake was recorded daily. From d 11 to 13 of each period, samples of each dietary ingredient were collected daily and mixed into one sample per period. Duodenal digesta was collected every 9 h during these 3 d. All samples were stored at -20°C .

Experiment 2

Cows and Treatments. Thirteen multiparous cows were assigned randomly to treatment sequence in a crossover design with a 14-d preliminary period followed by two 18-d treatment periods. The 2 treatments were diets containing either AL silage harvested at early to midbloom stage or OG silage harvested at early head stage as sole forage (Table 1). Both diets were formulated to contain 30% NDF and 25% forage NDF (Kammes and Allen, 2012). Consequently, the forage-to-concentrate ratio differed between diet, from 60:40 and 43:57 for AL and OG, respectively. Cows were fed ad libitum a TMR served once daily (1130 h) at 110% of expected intake.

Sample and Data Collection. Individual feed intake was recorded daily. From d 11 to 15 of each period, samples of each dietary ingredient were collected daily and mixed into 1 sample per period. Duodenal samples were collected every 15 h during these 5 d. All samples were stored at -20°C .

Laboratory Analyses. Frozen duodenal digesta samples were combined into one sample per cow per period, finely chopped using a commercial food processor (84142 Food cutter; Hobart Manufacturing Co., Troy, OH), and a representative subsample was lyophilized. Dietary ingredients were dried at 55°C in a forced-air oven for 72 h. All samples were ground with a Wiley mill (1-mm screen, Arthur H. Thomas, Philadelphia, PA).

Concentrations of B vitamins in feed and duodenal digesta were analyzed at the Sherbrooke Research and Development Centre (Sherbrooke, Québec, Canada). Thiamin, riboflavin, niacin, and vitamin B₆ were analyzed on HPLC (Varian Prostar, Lake Forest, CA) with a solvent delivery system (model 210), an autosampler (model 410), and a fluorescence detection system (model 363). Folate was analyzed with a microbiological microtiter plate test (VitaFast Folic Acid, R-Biopharm Inc., Marshall, MI), and vitamin B₁₂ was analyzed by radio-assay [SimulTRAC-S Vitamin B₁₂ (^{57}Co)/Folate (^{125}I), MP Biomedicals, Solon, OH]. All samples were analyzed in duplicates (except for folates which were analyzed in triplicates), and a coefficient of variation of less than 10% was accepted between replicates.

Thiamin. Thiamin concentrations were analyzed by HPLC according to a method adapted from Bötticher and Bötticher (1986). Standard curves were prepared as followed: a standard stock solution was prepared by dissolving 33.7 mg of thiamin hydrochloride (No. T4562, Sigma-Aldrich, Oakville, ON, Canada) in 100 mL of 0.1 M H₂SO₄ (No. 35129812, Fisher Scientific, Ottawa, ON, Canada). Then, 400 μL of the solution were 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, 0.6, 1.3, 2.6, 3.8, 5.0, and 7.5 μM). For the feed and digesta, 0.1 g of sample and 5 mL of 0.1 M 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 sodium acetate (No. S8750, Sigma-Aldrich) buffer and 500 μL of 1% reduced glutathione (No. G4251, Sigma-Aldrich) were added in 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 60 U of papain (No. P3250, Sigma-Aldrich), 10,167 U of α -amylase (No. A9857, Sigma-Aldrich), and 14 U of acid phosphatase (No. P3752, 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

Table 1. Ingredients, nutrient composition, and B-vitamin concentrations of the 2 treatment diets containing alfalfa silage (AL) or orchardgrass silage (OG) as sole forage in experiments 1 and 2¹

Item	Experiment 1		Experiment 2	
	AL	OG	AL	OG
Ingredient, % of DM				
Alfalfa silage	53.0 ²	—	59.9 ³	—
Orchardgrass silage	—	47.9 ⁴	—	42.7 ⁵
Dry ground corn	36.3	40.3	33.6	36.6
Soybean meal (48% CP)	6.50	7.00	—	11.8
Vitamin and mineral mix ^{6,7}	4.20	4.20	2.50	3.39
SoyPlus ⁸	1.30	1.30	3.99	3.99
Blood meal	0.30	0.90	—	—
Limestone	—	0.40	—	0.30
Urea	—	0.20	—	1.20
Nutrient				
DM, % as fed	43.6	50.6	54.5	52.3
OM, % of DM	91.5	91.5	92.7	91.1
NDF, % of DM	26.7	27.5	29.2	30.2
Forage NDF, % of DM	22.5	23.0	25.3	24.9
Indigestible NDF, % of DM	14.8	7.90	14.8	8.24
Starch, % of DM	30.2	32.1	27.3	29.6
CP, % of DM	18.3	18.8	18.4	17.0
B-vitamin concentrations, mg/kg of DM				
Thiamin	2.38	2.31	2.89	2.20
Riboflavin	36.93	46.07	66.95	20.50
Niacin ⁹	22.32	56.44	46.70	50.60
Vitamin B ₆ ¹⁰	17.63	11.84	7.38	3.36
Folates	0.36	0.46	0.36	0.44
Vitamin B ₁₂	ND ¹¹	ND	0.001	0.002

¹Adapted from Voelker Linton and Allen (2008) and Kammes and Allen (2012).

²DM = 30.6% (as fed); OM = 88.7%; NDF = 42.6%; indigestible NDF (iNDF) = 25.2%; potentially digestible NDF (pdNDF) = 17.4%; starch = 4.0%; CP = 20.5% (DM basis).

³DM = 43.5% (as fed); OM 91.9%; NDF = 42.3%; iNDF = 23.0%; pdNDF = 19.3%; starch = 1.87% = CP = 22.5% (DM basis).

⁴DM = 35.3% (as fed); and OM 89.2%; NDF = 48.0%; iNDF = 13.1%; pdNDF = 34.9%; starch = 2.3% = CP = 20.4% (DM basis).

⁵DM = 33.7% (as fed); and OM 90.3%; NDF = 58.2%; iNDF = 16.1%; pdNDF = 42.1%; starch = 1.37% = CP = 11.4% (DM basis).

⁶Experiment 1 = vitamin and mineral mix contained, on a DM basis, 11.7% dicalcium phosphate, 11.1% trace-mineral premix, 8.8% sodium bicarbonate, 2.3% magnesium oxide, 134.3 kIU/kg of vitamin A, 35.53 kIU/kg of vitamin D, 895.5 kIU/kg of vitamin E, and 65.2% ground corn grain as a carrier.

⁷Experiment 2 = vitamin and mineral mix contained, on a DM basis, 16.5% sodium bicarbonate, 14.2% magnesium sulfate, 7.1% salt, 5.8% dicalcium phosphate, 2.4% trace-mineral premix, 0.4% vitamin A, 0.4% vitamin D, 0.2% vitamin E, and 53.1% dry ground corn as a carrier.

⁸West Central Soy, Ralston, IA.

⁹Sum of the molar concentrations of niacin and nicotinamide; expressed as niacin equivalents.

¹⁰Sum of the molar concentrations of pyridoxamine, pyridoxal, and pyridoxine; expressed as pyridoxine equivalents.

¹¹ND = not detected.

mixed with 225 μL of oxidative solution [175 μL of 50% NaOH (No. S318, Fisher Scientific) and 50 μL of 5% potassium-ferricyanide (No. 1-3104, Baker, Avantor Performance Materials, Center Valley, PA) per sample]. After incubation (5 min), approximately 0.25 g of NaCl (No. S5886, Sigma-Aldrich) was added to each tube. Following mixing, 2 mL of 2-butanol (No. A398, Fisher Scientific) were added, the samples were shaken for 1 min, and then 10 μL were 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 potassium hydrogen phosphate (No. P5379, Sigma-Aldrich) buffer and 750 mL of acetonitrile (No. A998, Fisher Scientific) 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. The total HPLC run time required per sample was set to 7 min.

Riboflavin. Riboflavin concentrations were determined according to a method adapted from Giguère et al. (2002). Initial solutions of riboflavin (2,656 μM ; No. R4500, Sigma-Aldrich) and flavin-adenine dinucleotide (FAD; 2,411 μM ; No. F6625, Sigma-Aldrich) were used to obtain standard curves with 6 concentrations (5.3, 8.0, 13.3, 26.6, 53.1, and 79.7 μM and 5.3, 8.0, 13.3, 26.5, 53.0, and 79.6 μM for riboflavin and FAD, 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 (No. A144, 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 sodium acetate buffer and 500 μL of 1% reduced glutathione 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 completed to 15 mL with ultrapure water and centrifuged (900 $\times g$, 10 min, 4°C). The supernatant (500 μL) was combined with 200 μL of 15% perchloric acid (No. 77227, Sigma-Aldrich) 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 sodium acetate and 25 μL of 2% acid phosphatase [20 mg of phosphatase per mL of 50 mM ammonium acetate (No. 372331, Sigma-Aldrich) buffer (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 ammonium acetate 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 total HPLC run time required per sample was set to 8 min.

Niacin. Concentrations of the 2 forms of niacin, nicotinic acid (NA) and nicotinamide (NAM), were analyzed by HPLC with fluorimetric 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 (No. 72311, Sigma-Aldrich) and NAM (No. N5535, Sigma-Aldrich) in 100 mL of ultrapure water. Six concentrations of NA (5.0, 10.2, 20.3, 40.6, 81.2, and 100 μM) and NAM (5.2, 10.2, 20.5, 41.0, 81.9, and 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 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 sodium acetate buffer and 500 μL of 1% reduced glutathione were added in each sample. Next, the same trienzyme mix as used 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 supernatant (2 mL) was collected, of which 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 potassium dihydrogen phosphate (No. 60221, Sigma-Aldrich) buffer containing 0.075 M hydrogen peroxide (No. H325, Fisher Scientific) and 5.1 μM copper II sulfate (No. C1297, 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. The total HPLC run time required per sample was set to 14 min. Total niacin daily intake, duodenal flow, and ARS were the summation of NA and NAM for these variables.

Vitamin B₆. The 3 forms of vitamin B₆, pyridoxamine (PAM), pyridoxal (PAL), and pyridoxine (PYR), were determined by adaptation of methods described by Srivastava and Beutler (1973) and Matte et al. (1997). Four stock solutions made in ultrapure water (500 µg/mL) of PAM (No. P9380, Sigma-Aldrich), PAL (No. P9130, Sigma-Aldrich), PYR (No. P4722, Sigma-Aldrich), and pyridoxal-5'-phosphate (P5P; No. P9255, Sigma-Aldrich) were diluted (one-tenth for PAM, PAL, and P5P; one-thirtieth for PYR) to obtain standard solutions (207, 246, 202, and 81 µM, respectively). 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 H₂SO₄ were added to 0.2 g of sample and autoclaved (100°C, 15 min). After cooling in an ice bath, pH was adjusted to 4.5 with 4.0 M sodium acetate buffer, and 500 µL of 1% reduced glutathione were added in each tube. Next, adapted from the method of Hyun and Tamura (2005), 1 mL of an aqueous solution of 60 U of papain and 10,167 U of α-amylase 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 completed to 20 mL with ultrapure water and centrifuged (900 × 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 [20 mg of phosphatase per mL of ammonium acetate buffer (50 mM, pH 4.0)] to convert P5P into PAL. The samples were then centrifuged (10,000 × 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 of H₂SO₄ at a flow rate of 1.5 mL/min. Emission and excitation wavelengths of the fluorimetric detection system were 290 and 395 nm, respectively. The total HPLC run time required per sample was set to 10 min. 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 close to the level of detection, the mean intra-assay coefficient of variation were 4.5 and 3.4% in feed and digesta, respectively.

Folates. The method of extraction was adapted from AOAC International (2005, method 960.46) using the following extraction solution: 14.2 g of Na₂HPO₄ (No. S375, Fisher Scientific) and 10 g of citric acid (No. C0759, Sigma-Aldrich) in 1 L of ultrapure water (pH 7.3). In a 50-mL polypropylene conical tube, 5 mL of

this phosphate buffer were mixed with 0.1 g of solid material. According to the method adapted from Hyun and Tamura (2005), 100 µL of a papain solution (60 U) made in ultrapure water were 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, then 100 µL of α-amylase (10167 U) and 200 µL of conjugase (5 mg/mL, Pel-Freez Biologicals, Rogers, AR) solutions made in ultrapure water 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 × g, 10 min, 4°C) and the supernatants were used to determine folate concentration using a 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).

Vitamin B₁₂. The method of extraction was adapted from AOAC International (2005, 952.20) using the following extractive solution: 13 g of Na₂HPO₄, 12 g of citric acid, and 10 g of sodium metabisulfite (No. S244, 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 0.1 M NaCN (No. 205222, 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 6.2 to 6.5. The final volume was completed at 30 mL with ultrapure water and samples were centrifuged (3,000 × 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 B₁₂ using a commercial kit [SimulTRAC-S Vitamin B₁₂ (⁵⁷Co)/ Folate (¹²⁵I), MP Biomedicals].

Calculations and Statistical Analyses

The B vitamin concentrations in the TMR were the summation of B vitamin concentrations of each ingredient (Table 2) multiplied by the proportion of this ingredient in the TMR, on a DM basis. Daily intakes of each vitamin were calculated as the concentration of each vitamin in the TMR multiplied by the amount of TMR ingested, on a DM basis (Table 3). Calculations of DM duodenal flow (DF) were described by Voelker Linton and Allen (2008; experiment 1) and Kammes and Allen (2012; experiment 2). Duodenal flow of vitamins was calculated as B vitamin concentrations in duodenal digesta multiplied by the daily DM flowing through the

Table 2. B-vitamin concentrations (mg/kg of DM, unless noted) of ingredients used in the experimental diets

Vitamin	Alfalfa silage	Orchardgrass silage	Corn, dry ground	Soybean meal	SoyPlus ¹	Blood meal
Experiment 1						
Thiamin	1.96	1.73	3.35	2.31	1.11	0.21
Riboflavin	60.70	85.10	9.25	19.80	28.62	28.88
Niacin ²	21.60	92.80	20.50	48.70	67.16	56.28
Vitamin B ₆ ³	23.70	15.98	3.94	5.68	5.74	0.80
Folates	0.36	0.60	0.12	2.09	1.99	0.04
Vitamin B ₁₂ , µg/kg	ND ⁴	ND	ND	ND	ND	ND
Experiment 2						
Thiamin	2.70	1.23	3.59	2.92	0.64	—
Riboflavin	111	42.50	0.00	11.0	31.50	—
Niacin ²	56.20	68.10	30.20	55.6	117	—
Vitamin B ₆ ³	10.60	3.97	2.99	4.23	2.22	—
Folates	0.46	0.21	0.11	2.05	1.92	—
Vitamin B ₁₂ , µg/kg	1.15	3.00	1.30	1.30	1.11	—

¹West Central Soy, Ralston, IA.²Sum of the molar concentrations of niacin and nicotinamide; expressed as niacin equivalents.³Sum of the molar concentrations of pyridoxamine, pyridoxal, and pyridoxine; expressed as pyridoxine equivalents.⁴ND = not detected.

duodenum. Apparent ruminal synthesis was calculated as DF minus intake.

The data were analyzed separately for the 2 experiments. Daily intake, DF, and ARS for each vitamin, expressed in milligram per day or milligram per kilogram of DMI, were analyzed using the mixed procedure of

SAS (version 9.2; SAS Institute, 2008). The hypothesis that forage family affects responses of B vitamin daily intake, DF, and ARS was tested using the following model:

$$Y_{ijk} = \mu + C_i + P_j + T_k + PT_{jk} + \varepsilon_{ijk}$$

Table 3. Intake, duodenal flow, and apparent ruminal synthesis (ARS) of B vitamins (mg/d) in responses to diets based on alfalfa (AL) or orchardgrass (OG) silages (LS Means) in experiments 1 and 2

Vitamin	Experiment 1				Experiment 2			
	AL	OG	SEM	P-value	AL	OG	SEM	P-value
Thiamin								
Intake	49.8	46.2	2.86	0.23	69.2	51.1	2.00	<0.001
Duodenal flow	20.6	21.4	1.85	0.75	21.9	31.1	2.41	0.02
ARS	-29.2	-24.8	1.69	0.01	-46.5	-20.0	2.87	<0.001
Riboflavin								
Intake	771	920	51.4	0.03	1,633	476	45.0	<0.001
Duodenal flow	558	547	38.6	0.84	1,131	902	85.0	0.002
ARS	-214	-373	40.7	0.003	-501	426	71.0	<0.001
Niacin ¹								
Intake	466	1,128	53.6	<0.001	1,160	1,167	35.0	0.77
Duodenal flow	1,197	1,662	110.3	0.03	1,993	1,879	154.0	0.36
ARS	731	585	83.8	0.26	845	711	152.0	0.34
Vitamin B ₆ ²								
Intake	368	237	18.2	<0.001	180	77.3	5.13	<0.001
Duodenal flow	73.8	69.4	6.17	0.63	59.4	31.0	5.19	<0.001
ARS	-295	-167	14.5	<0.001	-121	-46.4	4.90	<0.001
Folates								
Intake	7.6	9.2	0.51	0.02	8.7	10.2	0.29	<0.001
Duodenal flow	28.1	37.8	4.16	0.15	38.9	39.8	2.66	0.78
ARS	20.5	28.6	3.85	0.19	30.2	29.5	2.50	0.81
Vitamin B ₁₂								
Intake	ND ³	ND	—	—	0.028	0.046	0.001	<0.001
Duodenal flow	10.1	10.0	0.89	0.93	12.68	9.92	0.90	0.01
ARS	10.1	10.0	0.89	0.93	12.65	9.97	0.90	0.02

¹Sum of the molar concentrations of niacin and nicotinamide; expressed as niacin equivalents.²Sum of the molar concentrations of pyridoxamine, pyridoxal, and pyridoxine; expressed as pyridoxine equivalents.³ND = not detected.

Table 4. Duodenal flow and apparent ruminal synthesis (ARS) of B vitamins (mg/kg of DMI) in responses to diets based on alfalfa (AL) or orchardgrass (OG) silages (LSM) in experiments 1 and 2

Vitamin	Experiment 1				Experiment 2			
	AL	OG	SEM	<i>P</i> -value	AL	OG	SEM	<i>P</i> -value
Thiamin								
Duodenal flow	0.98	1.11	0.045	0.09	1.58	2.24	0.179	0.024
ARS	−1.41	−1.24	0.049	0.05	−1.93	−0.83	0.104	<0.001
Riboflavin								
Duodenal flow	26.9	27.5	1.56	0.75	46.3	38.9	3.00	0.005
ARS	−10.0	−18.6	1.57	0.002	−21.1	18.4	3.03	<0.001
Niacin ¹								
Duodenal flow	58.3	83.0	4.07	0.002	82.0	80.6	5.93	0.79
ARS	36.0	29.3	4.02	0.28	34.6	30.0	6.23	0.40
Vitamin B ₆ ²								
Duodenal flow	3.55	3.46	0.200	0.76	2.41	1.35	0.182	0.003
ARS	−14.1	−8.38	0.200	<0.001	−5.02	−2.01	0.185	<0.001
Folates								
Duodenal flow	1.15	1.90	0.109	0.004	1.60	1.71	0.089	0.31
ARS	0.79	1.44	0.110	0.007	1.24	1.27	0.089	0.80
Vitamin B ₁₂								
Duodenal flow	0.49	0.51	0.038	0.71	0.51	0.42	0.028	0.020
ARS	0.49	0.51	0.038	0.71	0.51	0.42	0.027	0.020

¹Sum of the molar concentrations of niacin and nicotinamide; expressed as niacin equivalents.

²Sum of the molar concentrations of pyridoxamine, pyridoxal, and pyridoxine; expressed as pyridoxine equivalents.

where Y_{ijk} is the dependent variable, μ is the overall mean, C_i is the random effect of cow ($i = 1$ to 8, experiment 1; 1 to 13, experiment 2), P_j is the fixed effect of period ($j = 1$ to 2), T_k is the fixed effect of treatment ($k = 1$ to 2), PT_{jk} is the interaction of period and treatment, and ε_{ijk} is the residual error. Pearson correlation coefficients were determined using combined data from both experiments. Differences were declared significant at $P \leq 0.05$ and as a tendency at $0.05 < P \leq 0.10$.

RESULTS

Animal performance was presented and discussed previously by Voelker Linton and Allen (2008; experiment 1) and by Kammes and Allen (2012; experiment 2). Forage family did not affect neither DMI [experiment 1 = 20.9 vs. 20.0 (SE = 1.2; $P = 0.25$) kg/d; experiment 2 = 24.2 vs. 23.2 (SE = 0.63; $P = 0.13$) kg/d; for AL and OG, respectively] nor DM duodenal flow [experiment 1 = 12.6 vs. 13.7 (SE = 1.1; $P = 0.50$) kg/d; experiment 2: 14.9 vs. 13.9 (SE = 0.62; $P = 0.09$) kg/d; for AL and OG, respectively].

Vitamin Intake

Orchardgrass diets increased intakes of riboflavin and niacin in experiment 1, whereas it decreased riboflavin and increased thiamin intakes in experiment 2 ($P < 0.05$; Table 3). In both experiments, OG diet decreased vitamin B₆ intake and increased folate intake ($P < 0.05$; Table 3).

Apparent Ruminal Synthesis and Duodenal Flow

Apparent ruminal synthesis (expressed as mg/kg of DMI; Table 4) of thiamin, vitamin B₆, and folates was greater ($P < 0.05$) whereas riboflavin ARS was smaller ($P = 0.007$) for OG in experiment 1. However, in experiment 2, ARS of thiamin, riboflavin, and vitamin B₆ was greater ($P < 0.05$) whereas vitamin B₁₂ ARS was smaller ($P = 0.02$) for OG. Duodenal flow of niacin and folates (expressed as mg/kg of DMI; Table 4) was greater ($P < 0.05$) for OG in experiment 1, whereas DF of thiamin was smaller ($P = 0.02$) and riboflavin, vitamin B₆, and vitamin B₁₂ DF were greater ($P < 0.05$) for AL in experiment 2. Correlations between each B vitamin ARS and dietary and digestive parameters are reported in Table 5.

DISCUSSION

Plant maturity at harvest differed between the 2 experiments (experiment 1: early bud stage and early boot stage; experiment 2: early to midbloom and early head for AL and OG respectively). It is well known that nutrient composition of forage changes with advancing maturity (Jung and Allen, 1995; Yu et al., 2003; Yari et al., 2012), but little information is available on forage vitamin composition. In the present study, delaying harvest numerically decreased concentrations of all B vitamins in OG silage, whereas it increased thiamin, riboflavin, niacin, and folate concentrations but lowered vitamin B₆ concentrations in AL silage.

Calculation of ARS of B vitamins does not allow evaluating the confounded effects of microbial use, ruminal degradation, and potential absorption of B vitamins across the rumen wall. A lack of information exists on the extent of microbial use and degradation of B vitamins in the rumen, but some studies have shown that ruminal absorption is negligible. Hoeller et al. (1977) demonstrated the impermeability of the rumen wall to thiamin under in vitro conditions; however, according to Rrat et al. (1959), B vitamins in solution in an empty rumen can be absorbed across the rumen wall, but in fed animals, the amount absorbed from the rumen is negligible because B vitamins are mostly sequestered within bacteria. This observation is supported by findings from Santschi et al. (2005b), who reported that B vitamin concentrations are up to 5,000 fold higher in bacteria than in particle-free supernatant. Apparent ruminal synthesis probably underestimates the amounts of vitamins synthesized by ruminal microflora. Nevertheless, this calculated parameter allows comparisons among treatments and estimation of the relative importance of ruminal microflora for vitamin supply.

Thiamin

The amount of thiamin flowing to the duodenum was less than the daily intake, indicating losses in the

upper gastrointestinal tract, possibly due to degradation of the vitamin by the ruminal microflora. These losses were greater in AL diets, with 73% more thiamin degraded than with OG on average. Ruminal balance of thiamin varied from -1.03 to -1.67 mg/kg of DMI, on average, for OG and AL, respectively. These results differed from the values calculated from Castagnino et al. (2014; 4.35 mg/kg of DMI), Santschi et al. (2005a; 1.3 mg/kg of DMI), Schwab et al. (2006; 2.5 mg/kg of DMI), Breves et al. (1981; 3.72 mg/kg of DMI), and Steinberg and Kaufmann (1977; 5 mg/kg of DMI), all of which reported positive balance of ARS. Nevertheless, Miller et al. (1986) also observed ruminal degradation of thiamin (-1.30 mg/kg of DMI). Edwin and Jackman (1982) suggested that ruminal degradation of thiamin could be due to the presence of microbial thiaminase, which may be directly related to ruminal pH (Brent, 1976). However, when combining data from both experiments, thiamin ruminal degradation was not correlated with daily mean ruminal pH in the current studies (Table 5). In the present experiments, thiamin ARS was negatively correlated with OM intake and true ruminal degradation of OM, as well as with total VFA concentration, and tended to be correlated negatively with microbial N flow to duodenum. These correlations indicate that, under the present experimental conditions, increasing microbial biomass activity increased thiamin degradation in the rumen. On the contrary,

Table 5. Pearson correlations coefficients between apparent ruminal synthesis (ARS) of B vitamins and dietary and digestive parameters

Item	B-vitamin ARS (mg/d)					
	Thiamin	Riboflavin	Niacin ¹	Vitamin B ₆ ²	Folates	Vitamin B ₁₂
Intake, kg/d						
OM	-0.52***	-0.003	0.18	0.12	0.58***	0.54***
NDF	-0.49**	0.12	0.18	0.34*	0.57***	0.56***
iNDF	-0.78***	-0.51***	0.22	-0.29†	0.09	0.47**
Starch	-0.24	0.14	0.13	-0.04	0.59***	0.39*
N	-0.61***	-0.23	0.28†	0.04	0.57***	0.59***
Intake of vitamin, ³ mg/d	-0.78***	-0.74***	-0.19	-0.98***	0.59***	0.28†
Ruminally digested, kg/d						
True OM	-0.41**	0.002	-0.007	0.15	0.34*	0.30†
NDF	0.21	0.44**	-0.17	0.51***	0.29†	-0.01
Starch	-0.42	0.08	0.09	0.23	0.34*	0.29†
Ruminal measures						
VFA						
Total, mM	-0.34*	-0.06	-0.22	0.24	0.13	0.15
Acetate, mM	-0.21	0.26†	-0.29†	0.33*	0.02	0.10
Propionate, mM	-0.24	-0.01	-0.02	0.18	0.27†	0.08
Butyrate, mM	-0.25	-0.24	-0.30†	0.30†	0.09	0.20
Ruminal pH	-0.13	-0.29†	0.12	-0.49***	-0.22	-0.06
Flow to duodenum						
Microbial N, g/d	-0.27†	0.32*	0.49**	0.47**	0.58***	0.48**

¹Sum of the molar concentrations of niacin and nicotinamide; expressed as niacin equivalents.

²Sum of the molar concentrations of pyridoxamine, pyridoxal, and pyridoxine; expressed as pyridoxine equivalents.

³Intake of the corresponding B vitamin.

† $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Schwab et al. (2006) observed positive correlations between thiamin ARS and these variables. Daily thiamin intake in the present experiments varied from 46 to 69 mg/d and were in the higher range reported by Schwab et al. (2006), 27 to 46 mg/d. However, thiamin DF reported by Schwab et al. (2006) was more than 3 times greater than in the present experiments, 71 to 97 versus 21 to 31 mg/d, respectively, which explains the difference in ARS between these studies. The proportion of forage in diets used in experiments 1 (48 and 53%) and 2 (43 and 60%) were within the range studied by Schwab et al. (2006), who compared diets with 35 and 60% forage. Nevertheless, ingredient and nutrient compositions differed between these studies. For example, NDF concentrations were greater (35 to 47% of DM vs. 27 to 30% DM) but starch concentrations were smaller (5.2 to 20.7 vs. 27.3 to 32.1% of DM) in diets studied by Schwab et al. (2006) than in those used in experiments 1 and 2. Differences between the studies are probably due to nutrient composition and how it affects ruminal microorganism activity although the present results do not allow to identify the nutrient(s) explaining these differences.

Riboflavin

As for thiamin, we also observed a disappearance of riboflavin before the duodenum in experiment 1. In experiment 2 the AL treatment also presented a negative balance (-21.05 mg/kg of DMI), whereas in the OG treatment the ruminal balance was positive. Previous studies reported positive values for ARS (Santschi et al., 2005a, 13.48 mg/kg of DMI; Schwab et al., 2006, 11.69 mg/kg of DMI; and Miller et al., 1986, 8.88 mg/kg of DMI). Among the numerous differences in ingredient and nutrient composition of diets used in the studies cited above, it is noteworthy that in our study riboflavin intake was 8 to 38 fold greater than in those studies suggesting a regulatory mechanism by the ruminal microbiota. This hypothesis is supported by the observations that riboflavin ARS was negatively correlated with its intake and that a positive niacin ruminal balance was observed only for the treatment with the lowest vitamin intake (experiment 2, OG diet). Riboflavin ARS was positively correlated with ruminally digested NDF and microbial N flow to the duodenum. A trend for a positive correlation was also noted with the acetate concentration in the rumen and a negative correlation with the rumen pH. These facts may indicate that the fibrolytic bacteria are primarily responsible for the synthesis of riboflavin in the rumen.

Niacin

In spite of a marked difference in niacin intake in experiment 1, forage family did not affect niacin ARS in both experiments. Using grass-legume and corn silages as forages, Santschi et al. (2005a) reported niacin DF and ARS of 140 and 112 mg/kg of DMI, respectively. With diets containing corn silage, alfalfa, and grass hay, Schwab et al. (2006) observed niacin DF varying from 105 to 129 mg/kg of DMI, and ARS from 40 to 70 mg/kg of DMI. Those values are higher than observed in both current experiments. Contrary to the other studied vitamins, niacin ARS was not correlated with its intake. A positive correlation was noted between niacin ARS and microbial N flow to the duodenum, as previously observed by Schwab et al. (2006), and a positive trend with N intake. However, niacin ARS tended to be negatively correlated with concentrations of acetate and butyrate in the rumen. These correlations suggest that niacin ARS is increased when microbial biomass activity is raised.

Vitamin B₆

Around 63% of the vitamin B₆ ingested was used or degraded by the rumen microbes in experiment 2. This effect was greater and different between treatments (80 vs 71% for AL and OG, respectively) in experiment 1. Similarly, Santschi et al. (2005a) reported negative daily B₆ ARS (-0.71 mg/kg of DMI), whereas Schwab et al. (2006) observed positive ARS (1.13 mg/kg of DMI). As for thiamin and riboflavin, vitamin B₆ ARS was negatively correlated with its intake. Positive correlations were observed between vitamin B₆ ARS and NDF intake, NDF digested in the rumen, acetate and butyrate concentrations in the rumen, and microbial N flow to the duodenum. A negative correlation was noted between vitamin B₆ ARS and rumen pH. Synthesis of this vitamin in the rumen may therefore also be linked to fibrolytic bacterial activity.

Folates

When averaged across diets, daily folate ARS was 1.19 mg/kg of DMI, which is slightly greater than values reported by Santschi et al. (2005a; 1.06 mg/kg of DMI) and Schwab et al. (2006; ranging from 0.72 to 0.91 mg/kg of DMI). However, the proportion of folates in DF due to ARS observed by those authors was similar (ranging from 51 to 76%) to our experiments (75%, on average). The OG diet increased folate intake mainly due to the difference in soybean meal contribution between diets. Unlike thiamin, riboflavin, and

vitamin B₆, a positive correlation was observed between folate ingestion and its ARS. Folate ARS increased with nutrient (OM, NDF, starch, and N) intakes and the amounts digested in the rumen, as well as with ruminal propionate concentration and microbial N flow to the duodenum. Similar relationships were observed by Schwab et al. (2006), indicating that increasing the amount of substrates in rumen increases microbial folate synthesis.

Vitamin B₁₂

Unlike other B vitamins, vitamin B₁₂ is not synthesized by plants. Therefore, the amount of vitamin B₁₂ in diets is likely due to the bacterial synthesis occurring during silage fermentation or to contamination by soil microorganisms (McDowell, 2000). Production of vitamin B₁₂ in the rumen plays a very important role in cow nutrition because it represents, on average, 99% of the amount reaching the sites of absorption. Vitamin B₁₂ ARS varied from 0.42 to 0.51 mg/kg of DMI and was similar to 0.41 mg/kg of DMI reported by Castagnino et al. (2014). However, these values were much less than the average 3.69 and 3.93 mg/kg of DMI reported by Santschi et al., (2005a) and Schwab et al. (2006), respectively. As for folates, ARS of vitamin B₁₂ was positively correlated with nutrient intakes and microbial N flow to duodenum, which indicates that vitamin B₁₂ ARS is closely linked to microbial biomass activity.

CONCLUSIONS

Diets based on alfalfa silage resulted in a greater degradation of thiamin and vitamin B₆ in the rumen. Apparent ruminal synthesis of niacin was not affected by forage family, whereas the effect of family forage on ARS of riboflavin, folates, and vitamin B₁₂ differed between experiments. Based on the calculated correlations, fibrolytic microorganisms are likely involved in riboflavin and vitamin B₆ ARS. Moreover, ruminal microorganisms synthesized more or degraded less thiamin, riboflavin, and vitamin B₆ when the amount of vitamin ingested was limited, possibly in an attempt to fulfill their requirements. Apparent ruminal synthesis of folates and vitamin B₁₂ appear to be related to nutrient intake and microbial biomass activity. Results from the present experiments demonstrated that changes in diet composition affect the microbial population in the rumen and their B vitamin metabolism. Nevertheless, results are too limited to allow for the identification of the factor(s) responsible for these changes.

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