Cross-sectional study of the effect of diet composition on plasma folate and vitamin B₁₂ concentrations in Holstein cows in the United States and Canada

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ABSTRACT

The objective of this cross-sectional study was to assess the variability of plasma folate and vitamin B₁₂ concentrations in lactating Holstein cows across the United States and Canada. We also evaluated the effect of diet composition and cow characteristics on folate and vitamin B₁₂ plasma concentrations. A total of 22 and 24 US and Canadian dairy herds were enrolled, totaling 427 and 476 cows at 10 to 197 days in milk across all US and Canadian herds, respectively. Blood samples were taken to analyze plasma folate and vitamin B₁₂ concentrations, and ingredients of the diet were collected to determine nutrient composition. To reduce the number of interdependent variables in the analysis of the association of diet composition with plasma vitamin concentrations, we conducted a principal component analysis. Plasma folate concentrations were lower for US cows [13.4 ng/mL, 95% confidence interval (CI): 12.7–14.2] than for Canadian cows (14.5 ng/mL, 95% CI: 13.7–15.2), and the opposite was observed for plasma vitamin B₁₂ concentrations (US 206 pg/mL, 95% CI: 192–221; Canada 170 pg/mL, 95% CI: 159–181). The highest plasma concentrations of both vitamins were observed in the Northwest region of the United States (Oregon and Washington). Cows in California had the lowest plasma folate concentrations, and cows in Québec and New York State had the lowest plasma vitamin B₁₂ concentrations. Plasma folate concentrations were higher for multiparous than for primiparous cows and plasma vitamin B₁₂ concentrations progressively increased from parity 1 to 3 and higher. For both studied vitamins, plasma concentrations were lower at 0 to 55 than at 56 to 200 days in milk. Of 3 principal components, the one associated with dietary carbohydrates was significantly correlated

with plasma folate and vitamin B₁₂ concentrations. Indeed, plasma folate concentrations decreased with dietary fiber concentrations (i.e., neutral and acid detergent fibers and lignin) and increased with dietary nonfiber carbohydrate concentrations. We obtained the opposite results for plasma vitamin B₁₂ concentrations. Both multivariable models explained 41% (pseudo-R²) of the variation in plasma folate and vitamin B₁₂ concentrations. Information gathered in this study is the first step toward determining sources of variation in plasma folate and vitamin B₁₂ concentrations, as well as the vitamin status of cows.

Key words: cyanocobalamin, folic acid, principal component analysis, cattle

INTRODUCTION

In mammals, the roles of folate and vitamin B₁₂ in metabolism are closely related (Combs and McClung, 2017). Together, they play a crucial role in the methylation cycle, in which the methylated form of folates transfers its methyl group to homocysteine to form Met. The latter reaction requires vitamin B₁₂ as a coenzyme (Scott, 1999). This pathway allows the endogenous production of methyl donors, for which the requirement increases dramatically with milk production (Xue and Snoswell, 1985). Afterward, the demethylated form of folates can be used by the cells to provide 1-carbon units for synthesis of purine and pyrimidine, which are both constituents of DNA. Vitamin B₁₂ is also involved in converting propionate to succinyl CoA, which can then enter the Krebs cycle (Combs and McClung, 2017). This last reaction is of major importance for dairy cows, because about 60% of glucose in the blood circulation originates from propionate (Reynolds, 2006; Duplessis et al., 2017a). Moreover, folate status affects gene expression of the vitamin B₁₂-dependent enzyme in propionate metabolism (Preynat et al., 2010; Duplessis et al., 2017a).

Results from previous studies have shown that, given together to cows in early lactation, folic acid and

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vitamin B₁₂ supplements change energy partitioning. Indeed, combined supplement enhances production performance without modifying DMI and plasma indicators of body reserve mobilization (Graulet et al., 2007; Preynat et al., 2009a), or it decreases BW loss and plasma indicators of body reserve mobilization and increases plasma concentrations of glucose and insulin while maintaining milk production and DMI (Duplessis et al., 2014, 2017b). It is noteworthy that supplementing 1 of the 2 vitamins fails to elicit beneficial effects for early lactating cows when the status of the other vitamin is low (Girard et al., 2005; Akins et al., 2013); evidence for the interrelationship between these 2 vitamins is described above. The response to folic acid and vitamin B₁₂ supplementation seems to be driven by the animal's vitamin status (Duplessis et al., 2017b), which varies widely among studies (Graulet et al., 2007; Preynat et al., 2009b; Duplessis et al., 2017a). With the current state of knowledge, it is not possible to predict plasma vitamin concentrations without blood sampling and laboratory analysis. Little is known about the sources of variation in plasma folate and vitamin B₁₂ concentrations. Given the important roles played by these vitamins in mammals as described above, a better understanding of the causes of this variability could help in identifying cows that would benefit from folic acid and vitamin B₁₂ supplementation in early lactation.

It has been shown that ruminal bacteria can synthesize folates and vitamin B₁₂ (Bechdel et al., 1928). Findings from recent studies suggested that apparent ruminal syntheses (**ARS**) of folates and vitamin B₁₂ could be influenced by diet (Beaudet et al., 2016; Castagnino et al., 2017b; Seck et al., 2017). It has also been observed that ARS of folates were increased by high-starch and low-forage diets (Schwab et al., 2006; Beaudet et al., 2016; Seck et al., 2017). For vitamin B₁₂, Beaudet et al. (2016) showed that ARS was 200% greater with a high-fiber diet than with a low-fiber diet. Studying milk, Duplessis et al. (2019) concluded that variability in vitamin B₁₂ concentrations could partially be attributed to the cow's diet. Because folate and vitamin B₁₂ ARS are affected by diet composition, and a proportion of these synthesized vitamins are absorbed for metabolic functions, it can be hypothesized that diet would also affect folate and vitamin B₁₂ concentrations in plasma.

This study was first undertaken to determine the variability in plasma folate and vitamin B₁₂ concentrations in cows in early and mid lactation in commercial herds across the United States and Canada. A second objective was to assess the effect of diet composition and cow characteristics on plasma concentrations of these vitamins.

MATERIALS AND METHODS

Herds and Cows

We sent a letter inviting veterinarians ($n = 44$) from different regions of the United States and Canada to participate in this cross-sectional study. They were identified through business and university extension networking in the respective regions. The letter reviewed the work involved, outlined the objectives of the project, and described the expected outcomes. We collected expressions of interest in inclusion in the study by phone or email from January to March 2017. Participating veterinarians ($n = 13$) were asked to enroll dairy herds according to the requirements listed below; perform blood samplings on 10 healthy cows (i.e., without health disorders during the last 2 wk) per herd between 21 and 60 DIM and 10 healthy cows per herd between 100 and 180 DIM; collect each ingredient of the diet; provide diet printouts; and record sampled cow (i.e., parity and DIM) and herd (herd size, housing system, milk production) characteristics. We determined the sample size of 20 cows per herd for this study based on previous controlled trials involving 24 cows and on the variation known to exist in plasma folate and vitamin B₁₂ analysis between individuals [6 to 24 ng/mL for folates and 73 to 462 pg/mL for vitamin B₁₂, according to Preynat et al. (2009b) and Duplessis et al. (2017b)]. To participate in this study, US and Canadian herds were required to have at least 28 kg above the average milk production based on 305-d milk data from the USDA (2018) and Canadian Dairy Commission (2017), respectively, and they should have not been using recombinant bovine somatotropin. This information was verified from DHI or veterinarian records. Cows were excluded from the study if they were of a breed other than Holstein, if they had twinned, or if they had experienced a health disorder in the 2 wk before blood sampling. Veterinarians used a data sheet to record the answers to these questions and to enable the researchers to exclude cows' data if the answers warranted it. Diets given to the animals had to be served as a TMR or partial mixed ration and computed according to cow requirements based on a recognized software model, such as NRC (2001) or the Cornell Net Carbohydrate and Protein System. One participating herd gave intramuscular injections of vitamin B₁₂ to cows at less than 21 DIM; no cow from this herd was blood sampled at less than 29 DIM in this study. United States and Canadian veterinarians who performed blood sampling were committed to following the guidelines of the American Veterinary Medical Association (2019) and the Canadian Veterinary Medical Association (2019), respectively.

For this study, we enrolled a convenience sample of dairy herds across the United States and Canada. We aimed to enroll 5 herds per region to provide a snapshot of cows' status across the United States and Canada, and to obtain wide variability in diet ingredients and compositions: Northwest United States (Oregon, Washington, and Idaho); California; Southwest United States (Arizona, New Mexico, Nevada, and Texas); Upper Midwest United States (Michigan, Wisconsin, Minnesota, and Iowa); Northeast United States (New York, Pennsylvania, and Vermont); Southeast United States (Georgia, Florida, North Carolina, and Virginia); Québec, Canada; Ontario, Canada; Atlantic provinces, Canada (Nova Scotia and New Brunswick); Alberta, Canada; and British Columbia, Canada. We did not record information on how many herds were contacted by veterinarians and how many declined. Each herd was visited once by the veterinarian between March and December 2017, during which all required samples had to be collected.

Sample Collection and Analyses

One 10-mL blood sample was drawn from the coccygeal tail vein using a Vacutainer system (Becton, Dickinson and Co., Franklin Lakes, NJ) with EDTA tubes. Blood samples were put on ice for transportation and then centrifuged within 2 h of collection for 15 min at $3,100 \times g$ and 4°C. Plasma was harvested, stored at -20°C, and then sent to the Agriculture and Agri-Food Canada laboratory for analysis (Sherbrooke, QC, Canada). Plasma folate and vitamin B₁₂ concentrations were analyzed in duplicate by radioassay using a commercial kit (SimulTRAC B₁₂/FOLATE-S, MP Biomedicals, Solon, OH). The interassay coefficients of variation were 4.3 and 4.2% for the folate and vitamin B₁₂ analyses, respectively.

Each ingredient of the diet and TMR was sampled, and printouts of diets fed to the cows the day of the visit were kept. Veterinarians cooperated with the nutritionists to collect samples of individual ingredients and TMR. Representative samples were collected using the recommended sampling procedure outlined by the Soil and Forage Analysis Laboratory (Madison, WI). Feed samples were kept frozen until analyses. Then, samples were thawed at room temperature, dried using an air-forced oven at 55°C for 48 h, ground at 1 mm (Udy Cyclone Sample Mill, Fort Collins, CO), and analyzed by wet chemistry at Dairy One Cooperative Inc. (Ithaca, NY). Analytes obtained were CP [N $\times$ 6.25; method 990.03, AOAC International (2005)], ADF [Ankom (2017b), solutions as in method 973.18, AOAC Interna-

tional (2005)], aNDF [Ankom (2017c), solutions as in Van Soest et al. (1991) with the inclusion of heat-stable α -amylase and Na sulfite], lignin [Ankom (2017a), solutions as in method 973.18, AOAC International (2005)], fat [method 2003.05, AOAC International (2005)], ash [method 942.05, AOAC International (2005)], and Co [CEM (2019) using an inductively coupled plasma radial spectrometer]. We calculated NE_L and NFC [100 - (% NDF + % CP + % fat + % ash)] according to the NRC (2001). An aliquot of each ingredient was kept for folate concentration analysis at the Agriculture and Agri-Food Canada laboratory, as previously described by Beaudet et al. (2016), using a commercial microbiological microtiter plate test (Vita-Fast Folic Acid; R-Biopharm Inc., Marshall, MI). The interassay coefficient of variation was 3.89%. We did not analyze the vitamin B₁₂ concentrations of the ingredients; it was considered negligible (Beaudet et al., 2016; Castagnino et al., 2017a), because this vitamin is only synthesized by bacteria and is not naturally found in plants (Martens et al., 2002).

Calculations

Nutrient composition of diets was computed with Proc SQL in SAS (version 9.4; SAS Institute, 2012) using the nutrient composition of each ingredient multiplied by their respective DM percentage fed to the cows as described in diet printouts. Because some herds had several feeding groups, we matched the corresponding diet nutrient composition with the corresponding TMR fed to the cows. Percentages of corn silage, mixed silage (including grass, legume, or both, and barley silages), hay (including grass, legume, or both), nonforage fiber (including soybean hull, almond hull, or cottonseed), concentrate, cereal (including corn, oat, wheat, and barley), protein plant product (including soy and canola), commercial protein supplement, commercial energy supplement, fat supplement, and commercial mineral and vitamin supplement were computed by dividing the DM quantity of each ingredient by the DM quantity of the TMR fed to the animals as indicated in diet printouts. To be considered hay, the forage had to have a DM greater than 70%. Crude protein of greater than 30% was required for a commercial supplement to be categorized as a commercial protein supplement; otherwise, it was classified as a commercial energy supplement. Of the 46 participating herds, data from 11 herds could not be incorporated in the diet composition analysis, because important information was missing. Data from these herds were used only in the analysis of plasma folate and vitamin B₁₂ concentrations among

regions. For 10 additional herds, only the TMR nutrient composition was obtained; these herds were still considered in the diet composition analysis.

Statistical Analyses

We used Proc UNIVARIATE in SAS for descriptive statistics of herds. Proc MIXED of SAS was used to separately evaluate the difference in plasma folate and vitamin B₁₂ concentrations between countries as a fixed effect and herd as a random effect. If a finding was significant or a tendency, we then replaced country with region as a fixed effect in the model. Cow was the experimental unit. Residuals were visually assessed for normality and homoscedasticity. Normality assumption was violated for the plasma vitamin B₁₂ concentration analysis, and these data were log-transformed. Least squares means and 95% confidence intervals (95% CI) for the log-transformed dependent variable models were back-transformed using e^x , and then geometric means and 95% CI were presented. For uniformity, least squares means and 95% CI were presented even when data were not transformed. We obtained Cook's distance using the INFLUENCE option in Proc MIXED to identify and then suppress outlier data (Kaps and Lamberson, 2017). We deleted 26 and 3 plasma concentrations of folates and vitamin B₁₂ data, respectively, because they were considered influential.

We considered 10 independent continuous variables pertaining to diet composition [i.e., CP, ADF, aNDF, fat, ash, lignin, NFC (% of DM), NE_L (Mcal/kg of DM), Co (mg/kg of DM), and folates (μg/kg of DM)] in the analysis aiming to evaluate the relationship between cow diet composition and plasma concentrations of folates and vitamin B₁₂. To assess possible multicollinearity among variables, we used Proc CORR and Proc REG in SAS according to Kaps and Lamberson (2017) and Duplessis et al. (2019). A Spearman rank correlation coefficient (ρ) greater than 0.6 and a variance inflation factor greater than 10 suggest multicollinearity among independent variables. We found that ADF, aNDF, lignin, and NFC were highly correlated ($\rho > 0.7$), and the variance inflation factor for NFC was 15, suggesting multicollinearity. To overcome this issue, we used principal component analysis to reduce the number of interdependent variables in the analysis while considering most of the variation of the studied variables (Jolliffe, 2002). We performed Proc PRINCOMP in SAS using the standardized independent continuous variables to determine the number of principal components (PC) to retain in the model according to an eigenvalue greater than 1. Considering the number of PC previously determined, Proc FACTOR in SAS

with VARIMAX rotation allowed us to identify which variables made the largest contribution to a specific PC. To be included in a PC, the variable had to have the largest rotated pattern coefficient or loading value among the PC obtained.

We performed principal component regression analysis with Proc MIXED in SAS to evaluate the association between plasma concentrations of folates or vitamin B₁₂ and PC, parity, and DIM according to the following model (Atzori et al., 2013):

$$Y_{ij} = b_0 + b_1x_{1ij} + b_2x_{2ij} + b_3PC_{1ij} + b_4PC_{2ij} + \dots + b_nPC_{nij} + v_i + \varepsilon_{ij}$$

in which Y_{ij} is the plasma folate or vitamin B₁₂ concentration (dependent variables) of the j th cow of the i th herd; b_0 is the fixed intercept; b_1 and b_2 are the regression coefficients for parity and DIM, respectively, and x_{1ij} and x_{2ij} are the categories for parity (first, second, and third or greater) and DIM [0–55; 56–120; and 121–200 DIM] of the j th cow of the i th herd, respectively; b_3 is the regression coefficient of PC₁, and PC₁ is the score of the first PC of the j th cow of the i th herd, and so forth; v_i is the random intercept of the i th herd; and ε_{ij} is the Gaussian error term. We graphically assessed the linear relationship between dependent and independent variables using Proc GPLOT in SAS. Normality and homoscedasticity were graphically evaluated with residual distribution. The INFLUENCE option was also used in this mixed model; 11 and 9 records were considered influential and were deleted for plasma folate and vitamin B₁₂ concentrations, respectively. A Tukey's honest significant difference test was performed when categorical variable results reached significance. We obtained folate and vitamin B₁₂ plasma concentration predictions from the mixed multivariable models using the option OUTP in Proc MIXED. Afterward, we computed the pseudo-R² between predictions from the mixed models and observed plasma concentration values using Proc REG in SAS. Results were considered significant at $P \leq 0.05$ and a tendency when values were between 0.05 and 0.10.

Percentages of diet ingredients were not included in the principal component analysis because this would have required the suppression of 5 additional herds, resulting in the loss of important information needed for reliable results. The quantities fed of each ingredient in those herds were not provided, so the percentages of each ingredient in the diet could not be calculated. Nevertheless, we performed correlations between plasma concentrations in folates or vitamin B₁₂ and percentages of diet ingredients using Proc CORR in SAS to compute Spearman rank correlation coefficients.

RESULTS AND DISCUSSION

Descriptive Statistics and Differences Among Countries and Regions

A total of 46 Holstein herds (22 US and 24 Canadian herds) participated in this study from the following areas: Northwest (Oregon and Washington, $n = 2$); California ($n = 6$); Southwest (Arizona and Nevada, $n = 4$); Upper Midwest (Michigan and Wisconsin, $n = 5$); New York State ($n = 1$); Southeast (North Carolina and Virginia, $n = 4$); Québec, Canada ($n = 5$); Ontario, Canada ($n = 5$); Nova Scotia, Canada ($n = 5$); Alberta, Canada ($n = 4$); and British Columbia, Canada ($n = 5$). All cows were housed in tiestall ($n = 1$) or freestall ($n = 45$) barns. Herd sizes varied from 186 to 5,000, and from 85 to 444 cows for US and Canadian herds, respectively. The average milk production of US and Canadian herds was 38.0 kg/cow per day [standard deviation (SD) 3.9; range: 31.8–45.1] and 35.3 kg/cow/d (SD 3.3; range: 29.0–40.9), respectively. Average milk fat and true protein concentrations for US herds were 3.65% (SD 0.21; range: 3.24–4.09) and 3.15% (SD 0.15; range: 2.90–3.49), respectively. Average milk fat and crude protein concentrations for Canadian herds were 4.04% (SD 0.18; range: 3.70–4.40) and 3.28% (SD 0.11; range: 3.10–3.50), respectively. The average milk production and milk fat concentration of US herds were slightly higher and lower, respectively, than the national average (34 kg/d of milk and 3.84% fat; USDA, 2018). Milk production statistics for Canadian herds corresponded to the national average (34 kg/d of milk, 3.95% fat, and 3.25% of protein; Canadian Dairy Commission, 2017). Percentages of ingredients and the nutrient composition of diets for the studied regions are shown in Table 1. Because herds were not recruited based on dairy nutrition advisors, in some regions/provinces each herd had a different advisor, and in others all herds had the same advisor. For instance, British Columbia herds all had the same advisor, but Québec and New York herds had different advisors. Nevertheless, British Columbia herds showed good variability of ingredients and nutrient composition of diets (Table 1). Having the same advisor for all participating herds in this province did not decrease ration variability, probably because all herds used on-farm-produced forages and custom ingredients.

Four US cows were removed from the analysis because they were Jersey cows. As well, 9 US and 4 Canadian cows were removed due to misidentification. Hence, 427 US (148 first, 130 second, and 149 third or greater parity) and 476 Canadian (180 first, 115 second, and 164 third or greater parity; 17 unknown) Holstein dairy cows from 10 to 197 DIM were enrolled

in the current study. On average, the categories of 0–55, 56–120, and 121–200 DIM had 8.4 (SD 1.8), 4.5 (SD 2.7), and 6.8 (SD 2.3) cows per herd, respectively. Parity number was missing for 17 Canadian cows. Because only 1 herd was recruited in New York State, this herd was included in the closest region for this farm, which was the Canadian province of Québec (103 km from Montreal, QC, Canada).

Plasma concentrations of folates tended to be lower for US cows (13.4 ng/mL; 95% CI: 12.7–14.2) than for Canadian cows (14.5 ng/mL; 95% CI: 13.7–15.2; $P = 0.06$). Plasma vitamin B₁₂ concentrations were greater for US cows (206 pg/mL; 95% CI: 192–221) than for Canadian cows (170 pg/mL; 95% CI: 159–181; $P = 0.0002$). Differences among regions are depicted in Table 2. Plasma folate concentrations in the Northwest region were greater than in Ontario, the Upper Midwest, and California ($P = 0.04$). Cows from the Northwest region had greater plasma concentrations of vitamin B₁₂ than Canadian cows (including New York State), except those from Alberta ($P < 0.10$).

Principal Component Analysis

Principal component analysis allowed us to reduce the number of variables from 10 independent continuous variables to 3 PC (Table 3), explaining 71% of the total variation. The first, second, and third PC explained 43, 16, and 12% of the total variance, respectively. The first PC positively regrouped variables pertaining to percentages of folates and fiber (i.e., ADF, aNDF, lignin) in the diet and was negatively associated with the percentage of NFC of the diet. Concentrations of CP, Co, and ash in the diet were positively related to the second PC. The third PC was positively associated with concentrations of fat and NE_L in the diet. A PC can be considered an independent variable composed of a set of interrelated variables. Hence, a PC could be considered in the multivariable analysis as an independent variable that could be associated positively or negatively with the dependent variable (plasma folate or vitamin B₁₂ concentrations).

Relationships Between Folate and Vitamin B₁₂ Plasma Concentrations and Independent Variables

Folates. Plasma folate concentrations were affected by parity and DIM ($P < 0.0001$; Table 4). Plasma folate concentrations were lower in first parity (13.3 ng/mL; 95% CI: 12.6–14.0) than in second parity (14.8 ng/mL; 95% CI: 14.1–15.5) and third or greater parity (14.6 ng/mL; 95% CI: 13.9–15.3; $P \leq 0.0002$ for first vs. second and first vs. third or greater parity, and $P = 0.69$ for second vs. third or greater parity comparison).

Table 1. Average (SD), minimum, and maximum percentage of inclusion of each ingredient, and nutrient composition of diets in participating regions¹

Item	United States					Canada				
	Northwest	California	Southwest	Upper Midwest	Southeast	British Columbia	Alberta	Ontario	Québec and New York ²	Nova Scotia
Ingredient, %										
Forage and fiber sources										
Corn silage	27.2 (5.9)	10.6 (7.1)	15.3 (11.1)	35.6 (15.4)	—	28.9 (4.3)	18.9 (1.5)	27.3 (2.0)	42.1 (11.7)	23.9 (13.5)
Mixed silage ³	22.4–35.4	0.0–21.6	0.0–24.3	23.3–72.9	—	22.1–35.6	17.5–20.4	25.4–29.3	26.7–62.1	0.0–40.0
Hay ^{3,4}	7.0 (3.8)	9.9 (7.6)	27.4 (14.5)	20.5 (4.6)	—	20.5 (5.3)	14.6 (1.8)	31.1 (1.7)	16.6 (17.5)	32.7 (10.4)
Nonforage fiber ⁵	4.1–12.4	0.0–22.3	13.9–47.2	14.7–27.5	—	11.8–25.9	12.8–16.4	29.5–32.8	0.0–40.5	20.7–49.7
Energy source	12.9 (3.5)	10.8 (6.3)	15.9 (5.0)	0.0 (0.0)	—	5.8 (5.3)	23.4 (1.8)	0.9 (1.0)	6.8 (7.8)	1.0 (1.5)
Cereals ⁶	8.0–16.2	0.0–19.8	9.8–22.1	0.0–0.0	—	0.0–14.2	21.6–25.1	0.0–1.9	0.0–20.3	0.0–3.9
Commercial supplement and byproducts ^{7,8}	1.7 (2.3)	9.9 (8.5)	2.6 (3.6)	2.8 (4.9)	—	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Fat ⁹	0.0–4.9	0.0–23.9	0.0–7.6	0.0–11.5	—	0.0–0.0	0.0–0.0	0.0–0.0	0.0–0.0	0.0–0.0
Protein source	16.8 (3.2)	17.8 (9.0)	12.7 (9.0)	15.9 (12.0)	—	16.3 (11.7)	34.4 (4.4)	24.4 (4.8)	6.0 (6.8)	20.8 (5.1)
Soy and canola products	12.4–19.6	0.0–28.1	0.0–20.9	0.0–37.9	—	0.0–29.0	30.1–38.7	19.6–29.1	0.0–15.4	12.8–26.9
Commercial supplement ⁸	17.3 (13.3)	29.6 (24.5)	24.9 (5.7)	18.0 (16.2)	—	5.2 (5.2)	1.5 (1.6)	2.6 (2.6)	14.0 (10.6)	5.2 (4.7)
Vitamin and mineral supplements ¹⁰	6.5–36.1	2.6–61.4	20.0–33.0	0.0–43.7	—	0.0–12.2	0.0–3.1	0.0–5.2	0.0–25.8	0.0–11.4
Total concentrate ¹¹	0.0 (0.0)	0.4 (0.6)	0.6 (0.9)	0.0 (0.0)	—	0.5 (0.4)	0.0 (0.0)	0.3 (0.3)	0.0 (0.0)	0.8 (0.8)
Nutrient composition, % of DM, unless otherwise specified ¹²	0.0–0.0	0.0–1.3	0.0–2.0	0.0–0.0	—	0.0–0.9	0.0–0.0	0.0–0.6	0.0–0.0	0.0–2.1
CP	10.0 (7.1)	1.7 (3.2)	0.0 (0.0)	2.9 (5.1)	—	11.7 (9.6)	0.9 (0.9)	6.6 (6.7)	3.0 (4.2)	9.2 (6.0)
ADF	0.0–16.0	0.0–8.6	0.0–0.0	0.0–11.7	—	0.0–30.3	0.0–1.8	0.0–13.2	0.0–8.9	0.0–19.0
NDF	5.3 (7.6)	9.9 (13.2)	0.6 (0.8)	4.5 (7.9)	—	2.9 (4.0)	6.3 (6.4)	4.1 (4.2)	10.1 (10.0)	4.5 (6.5)
Lignin	0.0–16.1	0.0–37.4	0.0–1.7	0.0–18.4	—	0.0–9.6	0.0–12.6	0.0–8.2	2.4–24.1	0.0–16.5
NE _L , Mcal/kg of DM	1.5 (2.1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	—	0.0 (0.0)	0.0 (0.0)	2.2 (0.4)	1.4 (1.3)	1.5 (1.4)
NFC	0.0–4.3	0.0–0.0	0.0–0.0	0.0–0.0	—	0.0–0.0	0.0–0.0	1.8–2.6	0.0–3.2	0.0–3.7
Fat	52.7 (6.2)	68.8 (2.1)	41.3 (7.7)	43.9 (15.0)	—	44.8 (2.2)	43.1 (1.5)	40.6 (4.6)	34.5 (8.0)	42.4 (6.7)
Ash	44.3–58.6	66.5–71.9	35.0–52.2	9.6–61.5	—	41.1–47.6	41.7–44.5	36.0–45.2	17.5–44.8	32.3–50.3
CP	16.5 (1.1)	18.3 (1.1)	17.2 (0.7)	17.6 (2.0)	16.7 (1.4)	17.0 (2.6)	19.0 (0.0)	17.3 (0.9)	17.5 (2.3)	17.8 (0.8)
ADF	14.9–17.5	16.9–20.2	16.2–18.0	12.7–20.0	15.1–18.8	15.4–22.4	19.0–19.0	16.5–18.2	13.8–20.0	16.6–18.7
NDF	18.6 (3.2)	23.5 (1.8)	22.3 (4.9)	19.1 (2.0)	20.8 (1.7)	22.2 (2.2)	24.3 (0.0)	19.8 (0.2)	19.1 (2.7)	19.9 (2.5)
Lignin	14.4–22.1	20.2–25.8	16.5–28.3	17.7–23.1	19.2–23.4	19.8–26.2	24.3–24.3	19.6–20.0	16.2–22.8	16.8–24.5
NE _L , Mcal/kg of DM	28.1 (3.4)	34.4 (2.9)	30.6 (5.2)	29.3 (0.9)	31.9 (2.9)	34.1 (2.6)	35.7 (0.0)	31.5 (0.6)	30.6 (1.5)	31.6 (3.3)
NFC	23.4–31.7	29.3–39.4	23.9–36.5	27.7–30.5	29.2–36.4	32.3–39.5	35.7–35.7	30.9–32.1	28.5–32.3	28.7–37.4
Fat	3.4 (0.6)	6.1 (0.8)	4.3 (0.6)	3.4 (0.8)	2.6 (0.8)	3.8 (0.8)	5.3 (0.0)	3.7 (0.3)	2.9 (0.6)	3.4 (0.8)
Ash	2.6–4.1	5.2–7.5	3.7–5.0	2.3–4.7	1.5–3.6	3.0–5.4	5.3–5.3	3.4–4.0	2.2–3.6	1.9–4.2
NE _L , Mcal/kg of DM	1.60 (0.11)	1.62 (0.08)	1.57 (0.10)	1.72 (0.09)	1.71 (0.06)	1.64 (0.08)	1.74 (0.00)	1.66 (0.02)	1.74 (0.03)	1.64 (0.05)
NFC	1.46–1.71	1.50–1.74	1.44–1.69	1.58–1.85	1.65–1.80	1.50–1.74	1.74–1.74	1.64–1.68	1.70–1.77	1.58–1.71
Fat	36.1 (1.5)	34.6 (3.6)	37.9 (6.8)	40.4 (3.1)	40.0 (2.4)	35.1 (6.1)	28.0 (0.0)	39.3 (0.2)	39.8 (4.5)	36.1 (2.1)
Ash	34.2–37.8	27.4–38.5	31.8–47.4	36.8–47.4	36.6–43.2	22.7–40.2	28.0–28.0	39.1–39.5	35.4–46.3	33.2–38.7
NE _L , Mcal/kg of DM	4.5 (1.2)	5.0 (0.5)	4.1 (0.9)	4.8 (1.3)	3.9 (0.6)	4.5 (0.5)	8.5–8.5	4.0 (0.2)	4.7 (0.7)	4.5 (0.6)
NFC	3.2–6.2	4.0–5.6	3.2–5.3	3.6–7.5	3.1–4.6	4.0–5.4	8.5–8.5	3.8–4.2	3.8–5.5	3.9–5.5
Ash	6.8 (1.3)	8.3 (1.0)	10.0 (1.48)	7.9 (0.8)	7.5 (0.6)	9.6 (0.8)	8.9 (0.0)	5.1 (0.1)	5.9 (0.7)	7.2 (1.7)
NE _L , Mcal/kg of DM	5.5–8.7	6.5–9.7	8.2–11.9	6.0–9.1	6.6–8.0	8.6–10.6	8.9–8.9	4.9–5.2	4.9–6.8	5.3–10.1

Continued

Table 1 (Continued). Average (SD), minimum, and maximum percentage of inclusion of each ingredient, and nutrient composition of diets in participating regions¹

Item	United States					Canada				
	Northwest	California	Southwest	Upper Midwest	Southeast	British Columbia	Alberta	Ontario	Québec and New York ²	Nova Scotia
Co, mg/kg of DM	1.16 (0.67)	0.70 (0.22)	1.01 (0.71)	1.66 (0.5)	1.08 (0.35)	1.55 (0.20)	0.84 (0.00)	0.76 (0.15)	0.97 (0.43)	0.48 (0.09)
Folates, mg/kg of DM	0.50–2.08	0.40–1.09	0.32–1.99	1.12–2.40	0.77–1.62	1.33–1.83	0.84–0.84	0.61–0.91	0.60–1.56	0.36–0.62
	0.80 (0.09)	0.85 (0.09)	0.94 (0.21)	0.48 (0.06)	0.43 (0.05)	0.65 (0.09)	1.38 (0.00)	0.60 (0.00)	0.49 (0.20)	0.66 (0.20)
	0.67–0.88	0.71–0.98	0.78–1.25	0.39–0.54	0.35–0.50	0.55–0.78	1.38–1.38	0.60–0.60	0.28–0.75	0.46–1.01

¹Computed to meet dairy cow requirements according to lactation stage and milk production. Ingredients from the Southeast region of the United States were not available, and nutrient composition for Alberta province herds was obtained from 1 herd only.

²Because only 1 herd was enrolled in New York State, it was included in the closest region, which was Québec, Canada (103 km from Montreal, QC, Canada).

³Including grass, legume, or both, and barley silage.

⁴Forages with a DM >70%.

⁵Including soybean hull, almond hull, or cottonseed.

⁶Including corn, oats, wheat, and barley.

⁷Including commercial energy supplement, beet pulp, beet molasses, brewery, citrus, bakery byproducts, or a mixture.

⁸Nutrient compositions of commercial energy supplements and byproducts were (% of DM unless otherwise specified; $\pm$ SD): 17.0 $\pm$ 10.3% CP, 13.6 $\pm$ 8.1% ADF, 23.7 $\pm$ 13.2% NDF, 42.8 $\pm$ 19.7% NFC, 1.79 $\pm$ 0.20 Mcal of NE_L/kg of DM, 2.36 $\pm$ 3.99 mg/kg of DM Co, and 0.65 $\pm$ 1.21 mg/kg of DM of folates. Nutrient composition of commercial protein supplement was (% of DM unless otherwise specified): 45.3 $\pm$ 17.3% CP, 12.2 $\pm$ 8.1% ADF, 20.5 $\pm$ 12.5% NDF, 17.8 $\pm$ 7.8% NFC, 1.78 $\pm$ 0.35 Mcal of NE_L/kg of DM, 3.31 $\pm$ 4.90 mg/kg of DM Co, and 1.14 $\pm$ 0.96 mg/kg of DM of folates.

⁹Including rumen inert fat by supplying prilled hydrogenated or Ca soap fatty acids.

¹⁰For some herds, mineral and vitamin supplements were already included in the energy or protein commercial supplements.

¹¹Excluding forage categories such as corn and mixed silages, and hay.

¹²Obtained from nutrient composition of each ingredient multiplied by the percentage of the corresponding ingredient in the ration on a DM basis, or from the nutrient composition of the TMR.

Table 2. Plasma folate and vitamin B₁₂ concentrations in different regions of the United States and Canada

Region	Cows, n	Folates, ¹ ng/mL		Vitamin B ₁₂ , ^{2,3} pg/mL	
		Mean	95% CI	Mean	95% CI
United States					
Northwest	58	16.6 ^a	15.1–18.2	249 ^a	210–296
California	119	11.4 ^c	10.3–12.5	213 ^{ab}	189–241
Southwest	59	14.1 ^{ab}	12.6–15.6	211 ^{ab}	178–251
Upper Midwest	100	13.2 ^{bc}	11.8–14.5	186 ^{abc}	160–216
Southeast	71	14.1 ^{ab}	12.7–15.4	183 ^{abc}	157–213
Canada					
British Columbia	97	14.1 ^{ab}	12.9–15.3	170 ^{bc}	148–194
Alberta	79	15.2 ^{ab}	13.8–16.7	191 ^{abc}	163–224
Ontario	100	13.3 ^{bc}	12.1–14.5	179 ^{bc}	156–204
Québec and New York ⁴	120	15.7 ^{ab}	14.6–16.8	155 ^c	137–176
Nova Scotia	100	14.1 ^{ab}	12.9–15.3	166 ^{bc}	145–189

^{a–c}Means in the same row with different superscripts differ ($P < 0.10$).

¹Region effect, $P < 0.0001$.

²Geometric means and 95% CI for log-transformed data computed as e^x .

³Region effect, $P = 0.002$.

⁴Because only 1 herd was enrolled in New York State, it was included in the closest region, which was Québec, Canada (103 km from Montreal, QC, Canada).

Girard et al. (1995) also found that primiparous cows had lower serum folate concentrations than multiparous cows but Girard and Matte (1999) did not detect a parity effect. Because folates have a role in cell division (Combs and McClung, 2017), younger animals may use this vitamin for their own growth, lowering their plasma folate concentrations. Plasma folate concentrations were lower before 55 DIM (12.8 ng/mL; 95% CI: 12.1–13.4) than from 56 to 120 DIM (14.6 ng/mL; 95% CI: 13.9–15.4) or 121 to 200 DIM (15.3 ng/mL; 95% CI: 14.6–16.0; $P < 0.0001$). We noted no significant difference between 56 and 200 DIM ($P = 0.69$). Studies by Girard et al. (1989), Girard et al. (1995), and Girard and Matte (1999) observed a similar pattern during early lactation, with plasma folate concentrations highest at 56 DIM. Dairy cows face major metabolic challenges in early lactation, such as increased milk production combined with suboptimal DMI, often leading to negative energy balance; this could explain

why plasma folate concentrations were lower below 55 DIM. Moreover, increased milk production probably augments vitamin requirements.

The PC1 pertaining to dietary carbohydrates and folates was negatively associated with plasma folate concentrations ($P = 0.04$; Table 4). According to variable loadings of this PC shown in Table 3 and the negative association between plasma folate concentrations and PC1, folate concentrations in plasma were positively associated with the percentage of dietary NFC and negatively with the percentages of dietary ADF, aNDF, lignin, and folates. These results were in line with those of Schwab et al. (2006), Beaudet et al. (2016), and Seck et al. (2017), who reported that ARS of folates were decreased by fiber and increased by NFC or starch. The effect of dietary folate concentrations on folate ARS is conflicting in the literature. Beaudet et al. (2016) and Castagnino et al. (2017b) observed no significant association, and Castagnino et al. (2016a) and Seck et al. (2017) reported a positive correlation between intake and ARS of folates. Nevertheless, Hayes et al. (1966) found that ruminal fluid concentration of folates showed an inverse relationship concentrations in the diet. The current model explained 41% (i.e., pseudo- R^2) of the variation in plasma folate concentrations.

Vitamin B₁₂. Plasma concentrations of vitamin B₁₂ increased with parity ($P < 0.0001$; Table 4). Indeed, cows in their third parity or greater had greater plasma vitamin B₁₂ concentrations (210 pg/mL; 95% CI: 196–224) than first-parity cows (181 pg/mL; 95% CI: 166–196; $P < 0.0001$) and second-parity cows (199 pg/mL; 95% CI: 184–213; $P = 0.09$). Girard and Matte (1999) also reported that second-parity cows had high-

Table 3. Rotated pattern coefficients¹ of the principal component (PC) analysis

Variable	PC1	PC2	PC3
CP	33	64	10
ADF	88	20	–13
NDF	79	18	–3
Lignin	79	8	3
NE _L	–43	–20	80
NFC	–69	–54	8
Fat	46	19	81
Ash	45	60	4
Co	–35	80	–17
Folates	66	–3	23

¹Loadings multiplied by 100 and rounded to the closest integer.

er plasma vitamin B₁₂ concentrations than first-parity cows ($P = 0.01$). This finding was in contrast to those of Akins et al. (2013), who did not observe a parity effect on plasma vitamin B₁₂ concentrations. Similarly, vitamin B₁₂ concentrations in cow milk increased progressively with parity number (Duplessis et al., 2019). The same explanation for the parity effect of plasma folate concentrations prevailed for vitamin B₁₂, because this vitamin is also involved in cell division (Combs and McClung, 2017). Plasma concentrations of vitamin B₁₂ were greater between 56 and 200 DIM (205 pg/mL, 95% CI: 190–220 from 56 to 120 DIM; and 210 pg/mL, 95% CI: 196–225 from 121 to 200 DIM) than between 0 and 55 DIM (175 pg/mL; 95% CI: 161–188 pg/mL; $P < 0.0001$), as observed by Girard and Matte (1999), Preynat et al. (2009b), and Akins et al. (2013). We noted no difference from 56 to 120 and 121 to 200 DIM ($P = 0.61$). An explanation similar to the one for plasma folate concentrations could be made for the effect of lactation stage.

Only PC1 was positively correlated with plasma vitamin B₁₂ concentrations ($P = 0.04$; Table 4). Plasma concentrations of vitamin B₁₂ increased with the augmentation of diet concentrations of ADF, aNDF, lignin, and folates, and decreased with increases in dietary NFC concentration. Beaudet et al. (2016) obtained a 3-fold increment of vitamin B₁₂ ARS with a diet containing 49 vs. 36% NDF (DM basis), suggesting that fiber had a positive effect on ruminal synthesis of this vitamin. Moreover, several studies have shown that vitamin B₁₂ ARS was positively correlated with ADF and NDF (Schwab et al., 2006; Beaudet et al., 2016; Castagnino et al., 2016a,b) and negatively associated with NFC (Schwab et al., 2006). A recent trial study-

ing the effect of diet nutrient composition on vitamin B₁₂ concentrations in milk revealed that this vitamin was positively correlated with fiber concentration and negatively correlated with energy in the diet (i.e., NFC, starch, NE_L, and percentage of concentrate; Duplessis et al., 2019). Although current knowledge on ruminal bacteria species that synthesize vitamin B₁₂ is limited, these results suggest that species involved in ruminal fiber degradation are probably of major importance for vitamin synthesis. Dryden et al. (1962) detected that *Selenomonas ruminantium* was able to synthesize vitamin B₁₂; it has also been shown that this species could contribute to ruminal fiber digestion (Sawanon et al., 2011). The positive effect of dietary concentration of folates on plasma vitamin B₁₂ concentrations is difficult to explain, but it could be a consequence of the strong relationship between dietary folate and lignin percentages ($\rho = 0.61$; $P < 0.0001$). The model in the current study explained 41% (i.e., pseudo-R²) of the variation in plasma vitamin B₁₂ concentrations. Duplessis et al. (2019) concluded that their model explained 52% of the variation in vitamin B₁₂ concentrations in milk, including 46% of the variation explained by milk production. The remaining variation was explained by diet and animal characteristics. Plasma vitamin B₁₂ concentrations may be more closely related to cow nutrition than to milk vitamin B₁₂ concentrations. Moreover, Duplessis et al. (2018) showed that plasma and milk vitamin B₁₂ concentrations were not well correlated in Holstein cows, possibly explaining the discrepancy in pseudo-R² between those 2 studies.

The principal component including Co concentration in the diet (PC2) was not associated with plasma vitamin B₁₂ concentrations as previously reported for milk

Table 4. Relationships between plasma folate and vitamin B₁₂ concentrations and parity, DIM, and principal components (PC) following a mixed multivariable model analysis

Variable	Folates, ng/mL			Vitamin B ₁₂ , pg/mL		
	Estimate	SEM	P-value	Estimate	SEM	P-value
Intercept	11.86	0.38	<0.0001	158.76	7.61	<0.0001
Parity			<0.0001			<0.0001
First	0			0		
Second	1.49	0.33	<0.0001	17.95	6.41	0.005
Third or more	1.26	0.32	0.0001	29.41	6.18	<0.0001
DIM			<0.0001			<0.0001
0–55	0			0		
56–120	1.85	0.29	<0.0001	30.21	5.70	<0.0001
121–200	2.49	0.25	<0.0001	35.91	4.98	<0.0001
PC1 ¹	–0.68	0.31	0.04	13.64	6.29	0.04
PC2 ²	–0.07	0.30	0.82	2.33	6.23	0.71
PC3 ³	–0.01	0.30	0.97	–0.52	6.17	0.93

¹This PC was positively associated with dietary folate and fiber concentrations and negatively associated with dietary NFC concentration.

²This PC was positively associated with dietary CP, Co, and ash concentrations.

³This PC was positively associated with dietary fat and NE_L concentrations.

vitamin B₁₂ concentrations by Duplessis et al. (2016, 2019). The synthesis of vitamin B₁₂ by bacteria requires Co, because this trace metal is a constituent of the vitamin (Combs and McClung, 2017). Herds in previous studies and in the current study were offered rations containing higher Co concentration (0.19 to 2.36 mg/kg of DM) than the NRC (2001) recommendations (0.11 mg/kg of DM), and this could explain the absence of relationship between Co concentration in the diet and vitamin B₁₂ concentrations in plasma. The Spearman rank correlation coefficient between plasma vitamin B₁₂ concentrations and Co concentration in the diet was low ($\rho = 0.05$; $P = 0.16$; Figure 1a).

Relationships Between Plasma Folate and Vitamin B₁₂ Concentrations and Percentages of Diet Ingredients

Plasma folate and vitamin B₁₂ concentrations were not well correlated as suggested by the low Spearman rank correlation coefficient of 0.10 (Table 5 and Figure 1b).

Relationships between plasma folate and vitamin B₁₂ concentrations and percentages of ingredients in the diet were weak: Spearman rank correlation coefficients were below the absolute value of 0.28 (Table 5). These findings suggest that the ingredient composition of diets had a low effect on plasma folate and vitamin B₁₂ concentrations. Nevertheless, the relationship between folate or vitamin B₁₂ plasma concentrations and the percentage of corn silage followed an opposite trend, as suggested by the adverse signs of the correlation coefficients. The same conclusion could be made for non-forage fiber. Compared with hay, corn silage contains higher concentrations of NFC and lower concentrations of ADF, NDF, and lignin. Although corn silage was classified as a forage source in the current study, this ingredient generally contains a significant amount of grain. This could explain the inverse correlation between plasma vitamin B₁₂ concentrations and the percentage of corn silage in the diet, because this vitamin was negatively correlated with dietary NFC and positively correlated with dietary fiber. Beaudet et al. (2016) also observed that folate and vitamin B₁₂ ARS were increased by the opposite diet types: a high-starch ration increased folate ARS, and a high-fiber ration stimulated vitamin B₁₂ ARS. Seck et al. (2017) found that folate ARS was augmented by a low-forage diet, in contrast with vitamin B₁₂ ARS, which was higher with a high-forage diet. Similarly, Duplessis et al. (2019) found a positive relationship between commercial energy supplement and milk vitamin B₁₂ concentrations and a negative association between commercial protein supplement and milk vitamin B₁₂ concentrations.

Figure 1. Relationships between plasma vitamin B₁₂ concentrations in Holstein cows from 10 to 197 DIM and (a) Co concentration of diets (Spearman rank correlation coefficients = 0.05; $P = 0.16$) and (b) plasma folate concentrations (Spearman rank correlation coefficients = 0.10; $P = 0.004$).

It was interesting to highlight diets from the Northwest United States (highest plasma folate and vitamin B₁₂ concentrations), California (lowest plasma folate concentrations), and Québec and New York State (lowest plasma vitamin B₁₂ concentrations; Table 1). Compared with cows from the 2 other regions, cows from California were fed diets with the highest ADF, NDF, and lignin concentrations, and they had the lowest plasma folate concentrations. This was in line with the negative relationship between plasma folate concentrations and dietary fiber concentrations. In contrast, in Québec and New York State, cows were fed the highest dietary NFC concentrations and had the lowest average of plasma vitamin B₁₂ concentrations. In addition, the Northwest region of the United States had intermediate corn silage, nonforage fiber, and concentrate percentages in the diet compared to the 2 other regions. Importantly, although the studied vitamins were increased by the opposite diet characteristics, results for plasma

Table 5. Spearman rank correlation coefficients (*P*-value in parentheses) between plasma concentrations of folates and vitamin B₁₂ and percentages of ingredients in diets

Item	Folates, ng/mL	Vitamin B ₁₂ , pg/mL
Vitamin B ₁₂ , pg/mL	0.10 (0.004)	—
Ingredient, % of DM		
Forage and fiber sources		
Corn silage	0.23 (<0.0001)	−0.27 (<0.0001)
Mixed silage ¹	−0.004 (0.92)	−0.12 (0.002)
Hay ^{1,2}	0.06 (0.14)	0.21 (<0.0001)
Nonforage fiber ³	−0.28 (<0.0001)	0.21 (<0.0001)
Energy source		
Cereals ⁴	−0.11 (0.004)	0.04 (0.37)
Commercial supplement and byproduct ^{5,6}	−0.05 (0.28)	0.14 (0.0002)
Fat ⁷	−0.09 (0.02)	−0.04 (0.31)
Protein source		
Soy and canola products	0.04 (0.31)	−0.04 (0.34)
Commercial supplement ⁶	−0.01 (0.75)	−0.08 (0.05)

¹Including grass, legume, or both, and barley silage.

²Forages with a DM >70%.

³Including soybean hull, almond hull, or cottonseed.

⁴Including corn, oats, wheat, and barley.

⁵Including commercial energy supplement, beet pulp, beet molasses, brewery, citrus, bakery byproducts, or a mixture.

⁶Nutrient compositions of commercial energy supplements and byproducts were (% of DM unless otherwise specified; $\pm$ SD): 17.0 $\pm$ 10.3% CP, 13.6 $\pm$ 8.1% ADF, 23.7 $\pm$ 13.2% NDF, 42.8 $\pm$ 19.7% NFC, 1.79 $\pm$ 0.20 Mcal of NE_L/kg of DM, 2.36 $\pm$ 3.99 mg/kg of DM Co, and 0.65 $\pm$ 1.21 mg/kg of DM of folates. Nutrient composition of commercial protein supplement was (% of DM unless otherwise specified): 45.3 $\pm$ 17.3% CP, 12.2 $\pm$ 8.1% ADF, 20.5 $\pm$ 12.5% NDF, 17.8 $\pm$ 7.8% NFC, 1.78 $\pm$ 0.35 Mcal of NE_L/kg of DM, 3.31 $\pm$ 4.90 mg/kg of DM Co, and 1.14 $\pm$ 0.96 mg/kg of DM of folates.

⁷Including rumen inert fat by supplying prilled hydrogenated or Ca soap fatty acids.

folate and vitamin B₁₂ concentrations in the Northwest United States confirmed that it is possible to maximize both plasma vitamin concentrations.

Bach et al. (2008) concluded that 50% of milk yield variation was explained by non-dietary factors. Some non-dietary factors not measured in the current study could have explained a part of the variation in plasma folate and vitamin B₁₂ concentrations. One limitation of this study was that the quantity of each ingredient fed to the cow was obtained from diet printouts and was not compared with the actual quantity given by the dairy producer; the results from this study relied on the accuracy of diet printouts. Moreover, because separate ingredients were not collected for 10 herds, the ration could not be reconstituted and the nutrient composition of the TMR analyzed and taken for further analysis. Because a small numbers of herds were involved by region, the average nutrient composition for a given region was not necessarily typical of that region. Another limitation was that each herd was visited at 1 time point, so the studied ration was not necessarily representative of a year. Finally, the duration that the diet described on the printout was fed before the day of the visit was not recorded and may have been relatively short. These particularities should be kept in mind when interpreting the current results.

CONCLUSIONS

In the United States and Canada, marked regional differences in plasma concentrations of folates and vitamin B₁₂ were present. The highest plasma folate and vitamin B₁₂ concentrations were found in the Northwest region of the United States; the lowest plasma folate concentrations were observed in California, and the lowest plasma vitamin B₁₂ concentrations were found in Québec and New York State. Multivariable models explained 41% (pseudo-R²) of the plasma folate and vitamin B₁₂ concentrations. Parity and DIM were both factors that described the variation in plasma concentrations of those 2 vitamins. Among the studied dietary nutrient composition variables, plasma folate concentrations were negatively correlated with fiber concentrations and positively with NFC concentrations. The opposite was observed for plasma vitamin B₁₂ concentrations. Nevertheless, as supported by the low correlation coefficient between plasma concentrations of folates and vitamin B₁₂, a high status in both vitamins can be achieved, as was observed in cows from the Northwest United States. Knowledge obtained in the current study allowed for a better understanding of the sources of variation in plasma folate and vitamin B₁₂ concentrations, and is the first step toward identify-

ing cows that could benefit from folic acid and vitamin B₁₂ supplementation.

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REFERENCES

- Akins, M. S., S. J. Bertics, M. T. Socha, and R. D. Shaver. 2013. Effects of cobalt supplementation and vitamin B₁₂ injections on lactation performance and metabolism of Holstein dairy cows. *J. Dairy Sci.* 96:1755–1768. <https://doi.org/10.3168/jds.2012-5979>.
- American Veterinary Medical Association. 2019. Use of animals in research, testing, and education. Accessed Feb. 28, 2019. <https://www.avma.org/KB/Policies/Pages/Use-of-Animals-in-Research-Testing-and-Education.aspx>.
- Ankom. 2017a. Technology method 9—determining acid detergent lignin in Daisy incubator. Accessed Oct. 17, 2019. https://www.ankom.com/sites/default/files/document-files/Method_9_Lignin_in_Daisy.pdf.
- Ankom. 2017b. Technology method 12. ADF in feeds—filter bag technique. Accessed Aug. 1, 2019. https://www.ankom.com/sites/default/files/document-files/Method_12_ADF_A2000.pdf.
- Ankom. 2017c. Technology method 13. NDF in feeds—filter bag technique. Accessed Aug. 1, 2019. https://www.ankom.com/sites/default/files/document-files/Method_13_NDF_A2000.pdf.
- AOAC International. 2005. Official Methods of Analysis. 17th ed. AOAC International, Arlington, VA.
- Atzori, A. S., L. O. Tedeschi, and A. Cannas. 2013. A multivariate and stochastic approach to identify key variables to rank dairy farms on profitability. *J. Dairy Sci.* 96:3378–3387. <https://doi.org/10.3168/jds.2012-6256>.
- Bach, A., N. Valls, A. Solans, and T. Torrent. 2008. Associations between nondietary factors and dairy herd performance. *J. Dairy Sci.* 91:3259–3267. <https://doi.org/10.3168/jds.2008-1030>.
- Beaudet, V., R. Gervais, B. Graulet, P. Nozière, M. Doreau, A. Fanchone, D. S. Castagnino, and C. L. Girard. 2016. Effects of dietary nitrogen levels and carbohydrate sources on apparent ruminal synthesis of some B vitamins in dairy cows. *J. Dairy Sci.* 99:2730–2739. <https://doi.org/10.3168/jds.2015-10521>.
- Bechdel, S. I., H. E. Honeywell, R. A. Dutcher, and M. H. Knutsen. 1928. Synthesis of vitamin B in the rumen of the cow. *J. Biol. Chem.* 80:231–238.
- Canadian Dairy Commission. 2017. The industry. Accessed Nov. 1, 2019. <http://www.cdc-ccl.gc.ca/CDC/index-eng.php?id=3796>.
- Canadian Veterinary Medical Association. 2019. Use of animals in science—position statement. Accessed Feb. 28, 2019. <https://www.canadianveterinarians.net/documents/use-of-animals-in-science-position-statement>.
- Castagnino, D. S., K. J. Harvatine, M. S. Allen, R. Gervais, P. Y. Chouinard, and C. L. Girard. 2017a. Short communication: Effect of fatty acid supplements on apparent ruminal synthesis of B vitamins in lactating dairy cows. *J. Dairy Sci.* 100:8165–8169. <https://doi.org/10.3168/jds.2017-13087>.
- Castagnino, D. S., K. L. Kammes, M. S. Allen, R. Gervais, P. Y. Chouinard, and C. L. Girard. 2016a. Particle length of silages affects apparent ruminal synthesis of B vitamins in lactating dairy cows. *J. Dairy Sci.* 99:6229–6236. <https://doi.org/10.3168/jds.2016-11274>.
- Castagnino, D. S., K. L. Kammes, M. S. Allen, R. Gervais, P. Y. Chouinard, and C. L. Girard. 2017b. High-concentrate diets based on forages harvested at different maturity stages affect ruminal synthesis of B vitamins in lactating dairy cows. *Animal* 11:608–615. <https://doi.org/10.1017/S1751731116001798>.
- Castagnino, D. S., M. Seck, V. Beaudet, K. L. Kammes, J. A. Voelker Linton, M. S. Allen, R. Gervais, P. Y. Chouinard, and C. L. Girard. 2016b. Effects of forage family on apparent ruminal synthesis of B vitamins in lactating dairy cows. *J. Dairy Sci.* 99:1884–1894. <https://doi.org/10.3168/jds.2015-10319>.
- CEM. 2019. MARS 6. Method note compendium. Accessed Oct. 17, 2019. <http://cem.com/en/mars-6-method-note-compendium>.
- Combs, G. F. Jr., and J. P. McClung. 2017. The vitamins. Fundamental aspects in nutrition and health. 5th ed. Academic Press, Oxford, United Kingdom.
- Dryden, L. P., A. M. Hartman, M. P. Bryant, I. M. Robinson, and L. A. Moore. 1962. Production of vitamin B₁₂ and vitamin B₁₂ analogues by pure cultures of ruminal bacteria. *Nature* 195:201–202. <https://doi.org/10.1038/195201b0>.
- Duplessis, M., R. I. Cue, D. E. Santschi, D. M. Lefebvre, and C. L. Girard. 2018. Short communication: Relationships among plasma and milk vitamin B₁₂, plasma free fatty acids, and blood β -hydroxybutyrate concentrations in early lactation dairy cows. *J. Dairy Sci.* 101:8559–8565. <https://doi.org/10.3168/jds.2018-14477>.
- Duplessis, M., C. L. Girard, D. E. Santschi, D. M. Lefebvre, and D. Pellerin. 2014. Milk production and composition, and body measurements of dairy cows receiving intramuscular injections of folic acid and vitamin B₁₂ in commercial dairy herds. *Livest. Sci.* 167:186–194. <https://doi.org/10.1016/j.livsci.2014.06.022>.
- Duplessis, M., H. Lapierre, B. Ouattara, N. Bissonnette, D. Pellerin, J. P. Laforest, and C. L. Girard. 2017a. Whole-body propionate and glucose metabolism of multiparous dairy cows receiving folic acid and vitamin B₁₂ supplements. *J. Dairy Sci.* 100:8578–8589. <https://doi.org/10.3168/jds.2017-13056>.
- Duplessis, M., H. Lapierre, D. Pellerin, J. P. Laforest, and C. L. Girard. 2017b. Effects of intramuscular injections of folic acid, vitamin B₁₂, or both, on lactational performance and energy status of multiparous dairy cows. *J. Dairy Sci.* 100:4051–4064. <https://doi.org/10.3168/jds.2016-12381>.
- Duplessis, M., D. Pellerin, R. I. Cue, and C. L. Girard. 2016. Short communication: Factors affecting vitamin B₁₂ concentration in milk of commercial dairy herds: An exploratory study. *J. Dairy Sci.* 99:4886–4892. <https://doi.org/10.3168/jds.2015-10416>.
- Duplessis, M., D. Pellerin, R. Robichaud, L. Fadul-Pacheco, and C. L. Girard. 2019. Impact of diet management and composition on vitamin B₁₂ concentration in milk of Holstein cows. *Animal* 13:2101–2109. <https://doi.org/10.1017/S1751731119000211>.
- Girard, C. L., H. Lapierre, J. J. Matte, and G. E. Lobley. 2005. Effects of dietary supplements of folic acid and rumen-protected methionine on lactational performance and folate metabolism of dairy cows. *J. Dairy Sci.* 88:660–670. [https://doi.org/10.3168/jds.S0022-0302\(05\)72730-2](https://doi.org/10.3168/jds.S0022-0302(05)72730-2).
- Girard, C. L., and J. J. Matte. 1999. Changes in serum concentrations of folates, pyridoxal, pyridoxal-5-phosphate and vitamin B₁₂ during lactation of dairy cows fed dietary supplements of folic acid. *Can. J. Anim. Sci.* 79:107–114. <https://doi.org/10.4141/A98-016>.
- Girard, C. L., J. J. Matte, and G. F. Tremblay. 1989. Serum folates in gestating and lactating dairy cows. *J. Dairy Sci.* 72:3240–3246. [https://doi.org/10.3168/jds.S0022-0302\(89\)79483-2](https://doi.org/10.3168/jds.S0022-0302(89)79483-2).
- Girard, C. L., J. J. Matte, and G. F. Tremblay. 1995. Gestation and lactation of dairy cows: A role for folic acid? *J. Dairy Sci.* 78:404–411. [https://doi.org/10.3168/jds.S0022-0302\(95\)76649-8](https://doi.org/10.3168/jds.S0022-0302(95)76649-8).
- Graulet, B., J. J. Matte, A. Desrochers, L. Doepel, M.-F. Palin, and C. L. Girard. 2007. Effects of dietary supplements of folic acid and vitamin B₁₂ on metabolism of dairy cows in early lactation. *J. Dairy Sci.* 90:3442–3455. <https://doi.org/10.3168/jds.2006-718>.
- Hayes, B. W., G. E. Mitchell Jr., C. O. Little, and N. W. Bradley. 1966. Concentrations of B-vitamins in ruminal fluid of steers fed

- different levels and physical forms of hay and grain. *J. Anim. Sci.* 25:539–542. <https://doi.org/10.2527/jas1966.252539x>.
- Jolliffe, I. T. 2002. *Principal component analysis*. 2nd ed. Springer-Verlag, New York, NY.
- Kaps, M., and W. R. Lamberson. 2017. *Biostatistics for Animal Science*. 3rd ed. CAB International, London, UK.
- Martens, J.-H., H. Barg, M. Warren, and D. Jahn. 2002. Microbial production of vitamin B₁₂. *Appl. Microbiol. Biotechnol.* 58:275–285. <https://doi.org/10.1007/s00253-001-0902-7>.
- NRC. 2001. *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Preynat, A., H. Lapierre, M. C. Thivierge, M. F. Palin, N. Cardinault, J. J. Matte, A. Desrochers, and C. L. Girard. 2010. Effects of supplementary folic acid and vitamin B₁₂ on hepatic metabolism of dairy cows according to methionine supply. *J. Dairy Sci.* 93:2130–2142. <https://doi.org/10.3168/jds.2009-2796>.
- Preynat, A., H. Lapierre, M. C. Thivierge, M. F. Palin, J. J. Matte, A. Desrochers, and C. L. Girard. 2009a. Effects of supplements of folic acid, vitamin B₁₂, and rumen-protected methionine on whole-body metabolism of methionine and glucose in lactating dairy cows. *J. Dairy Sci.* 92:677–689. <https://doi.org/10.3168/jds.2008-1525>.
- Preynat, A., H. Lapierre, M. C. Thivierge, M. F. Palin, J. J. Matte, A. Desrochers, and C. L. Girard. 2009b. Influence of methionine supply on the response of lactational performance of dairy cows to supplementary folic acid and vitamin B₁₂. *J. Dairy Sci.* 92:1685–1695. <https://doi.org/10.3168/jds.2008-1572>.
- Reynolds, C. K. 2006. Production and metabolic effects of site of starch digestion in dairy cattle. *Anim. Feed Sci. Technol.* 130:78–94. <https://doi.org/10.1016/j.anifeedsci.2006.01.019>.
- SAS Institute. 2012. *User's Guide: Statistics*. Version 9.4. SAS Institute, Cary, NC.
- Sawanon, S., S. Koike, and Y. Kobayashi. 2011. Evidence for the possible involvement of *Selenomonas ruminantium* in rumen fiber digestion. *FEMS Microbiol. Lett.* 325:170–179. <https://doi.org/10.1111/j.1574-6968.2011.02427.x>.
- Schwab, E. C., C. G. Schwab, R. D. Shaver, C. L. Girard, D. E. Putnam, and N. L. Whitehouse. 2006. Dietary forage and nonfiber carbohydrate contents influence B-vitamin intake, duodenal flow, and apparent ruminal synthesis in lactating dairy cows. *J. Dairy Sci.* 89:174–187. [https://doi.org/10.3168/jds.S0022-0302\(06\)72082-3](https://doi.org/10.3168/jds.S0022-0302(06)72082-3).
- Scott, J. M. 1999. Folate and vitamin B₁₂. *Proc. Nutr. Soc.* 58:441–448. <https://doi.org/10.1017/S0029665199000580>.
- Seck, M., J. A. Voelker Linton, M. S. Allen, D. S. Castagnino, P. Y. Chouinard, and C. L. Girard. 2017. Apparent ruminal synthesis of B vitamins in lactating dairy cows fed diets with different forage-to-concentrate ratios. *J. Dairy Sci.* 100:1914–1922. <https://doi.org/10.3168/jds.2016-12111>.
- USDA-National Agricultural Statistics Service. 2018. Milk production, disposition, and income: 2017 summary. Accessed Dec. 14, 2019. <https://downloads.usda.library.cornell.edu/usda-esmis/files/4b29b5974/4j03d209m/qr46r324g/MilkProdDi-04-26-2018.pdf>.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74:3583–3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2).
- Xue, G. P., and A. M. Snoswell. 1985. Regulation of methyl group metabolism in lactating ewes. *Biochem. Int.* 11:381–385.

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