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Invited Review

New approaches, development, and improvement of methodologies for the assessment of B-vitamin requirements in dairy cows

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ABSTRACT - Studies on B-vitamin requirements of cattle, conducted more than 60 years ago, concluded that mature ruminants do not require B-vitamin supplements because the amounts of vitamins provided by the diet and synthesized by the ruminal microflora were sufficient to prevent emergence of deficiency symptoms. As a result, the impact of subclinical deficiency on maintenance of normal and efficient metabolism has been disregarded and very little research effort has been devoted at defining dairy cow requirements for B vitamins. However, emergence of deficiency symptoms is the last stage of the deficiency; deficiency appears as soon as the supply is inferior to the needs, leading to a loss of metabolic efficiency. As B vitamins play critical roles in carbohydrate, protein, and lipid metabolism, it is likely that the demand for these cofactors increases with milk yield. Reports over the last two decades of beneficial effects of B-vitamin supplementation, such as thiamin, niacin, biotin, folic acid, and vitamin B_{12}, suggested that, under some conditions, the need for B vitamins exceeds the supply from the diet and the synthesis by rumen microbes, leading to sub-optimal milk production and metabolic efficiency. However, responses to B-vitamin supplementation are highly variable. The major challenge faced by studies on B-vitamin requirements of dairy cows is the very limited knowledge on dietary factors driving the fate of B vitamins in rumen. This knowledge is essential to identify the conditions under which the dairy cow could benefit from B-vitamin supplements. The present review aims to describe the present state of knowledge on B-vitamin requirements of dairy cows as well as some of the major problems that need to be overcome to progress in this research field.

Key Words: B-vitamin, dairy cow, metabolism, requirement, rumen

Introduction

Vitamins were discovered relatively recently, during the first half of the 20th century. Vitamins are organic compounds, present in minute amounts in foods and feedstuffs, although they are also needed in minute amounts for maintenance of physiological functions. They do not have a structural function and their catabolism does not provide a significant source of energy. With a few exceptions, vitamins are not synthesized by the tissues of the host in quantities sufficient to meet normal physiological needs (Combs, 2012). Their nomenclature is strictly empirical; the eight B vitamins share no chemical or functional similarities. Nevertheless, all of them are involved in at least one of the three following major biological functions: coenzymes, required for enzyme activity; oxidation-reduction reactions; and gene transcription.

Balancing dairy cow diets: why overlook B vitamins for decades?

Already in 1926, in a time when only one B vitamin, thiamin, had been identified, Bechdel and his collaborators published results from a series of studies aiming to determine cattle requirements for B vitamin. In all these studies, starting after weaning, the animals fed diets based on byproducts assumed to be low in B vitamin and using beet pulp as the only source of fibre. The diets were tested on rats, which rapidly developed B vitamin deficiency symptoms. Calves raised on these diets deficient in B vitamin grew normally, were successfully bred, calved, and produced milk. Consequently, the authors concluded that "the livestock man need have little concern about vitamin B deficiency ever standing in the way of his raising strong, healthy calves" (Bechdel et al., 1926).

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Two years later, Bechdel et al. (1928) cleverly demonstrated that B vitamins are produced by microorganisms present in the rumen. They compared the growth of rats fed either rumen content from cows fed a “B-vitamin deficient” diet or the cow diet itself. Growth of rats fed the latter was rapidly impaired, whereas those fed rumen content thrived. Since then, it was generally recognized that mature ruminants do not require B-vitamin supplements because the amounts of vitamins provided by the diet and synthesized by the ruminal microflora were sufficient to prevent emergence of deficiency symptoms.

Nevertheless, Bechdel et al. (1926) reported an interesting observation: all their cows stopped eating two weeks after calving, some of them died, whereas others recovered only when returned to a normal standard diet. The authors commented briefly this observation and related the problem to a lack of fibre. This observation raises, however, an intriguing question: if these diets did not allow for an optimal rumen function, could they have limited the synthesis of B vitamins to quantities insufficient to sustain the increased demand due to colostrum and milk production?

B-vitamin supplements for dairy cows: what can we learn from their use?

Emergence of deficiency symptoms is the last stage of the deficiency, which appears as soon as the supply is inferior to the needs leading rapidly to a loss of metabolic efficiency (Combs, 2012). As B vitamins play critical roles in carbohydrate, protein, and lipid metabolism, it is likely that the demand for these cofactors increases with milk yield. Moreover, after calving, colostrum production rapidly drains large amounts of B vitamins; concentrations of most B vitamins are greater in colostrum than milk (Duplessis et al., 2015; McGrath et al., 2016). For example, folate and vitamin B_{12} concentrations were six and ten times greater in colostrum than milk, respectively (Duplessis et al., 2015). The absence of deficiency symptoms does not preclude the presence of a subclinical deficiency and its negative impact on maintenance of normal and efficient metabolism. Interestingly, there is evidence in the literature that dairy cows could benefit from B-vitamin supplements. For some of the vitamins, periods or conditions of increased requirements have been identified. The section below will briefly summarize some of these observations.

Thiamin

Thiamin is a coenzyme for enzymatic complexes involved in carbohydrate and energy metabolism. Shaver and Bal (2000) described the responses of dairy cows to thiamin dietary supplements, at doses of 150 or 300 mg day^{-1}, in three trials. When comparing results from these trials, the authors concluded that increases in milk production and milk fat and protein yields following use of thiamin supplements were observed only when cows were fed diets with low neutral detergent fiber concentrations and high concentrations of non-fiber carbohydrates.

Niacin

Niacin is the essential component of nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate involved in all reduction-oxidation reactions. As such, these two molecules play a crucial role in carbohydrate, fatty acid, and amino acid metabolism. According to a meta-analysis (Schwab et al., 2005) using data from 27 studies published between 1980 and 1998, a dietary supplement of 12 g day^{-1} of niacin increases milk fat yield and tends to increase 3.5% fat-corrected milk and protein yield. As there is no effect of the vitamin supplement on dry matter intake, feed efficiency calculated with 3.5% fat-corrected milk tends also to be increased. Nevertheless, even if niacin is the most extensively studied B vitamin in dairy cows, production and metabolic responses to niacin supplements are highly variable (Flachowsky, 1993).

Biotin

Biotin plays key roles in lipid, amino acid, and energy metabolism due to its function as coenzyme for five enzymes. Two of them are of special interest for ruminants due to their importance for gluconeogenesis. Ferreira et al. (2007) stressed that supplementary biotin was more likely to increase milk and milk component yields in high-producing cows than in low-producing ones because the metabolic demand was greater in the former. Nevertheless, in some studies, high-producing cows did not respond to biotin supplementation (Rosendo et al., 2004). In 2011, two meta-analyses on the effects of dietary supplements of biotin on milk production and composition of Holstein dairy cows were published (Chen et al., 2011; Lean and Rabiee, 2011). Both meta-analyses used data from 11 comparisons, but with only six comparisons were common to both studies; exclusion and inclusion criteria also differed between analyses. In spite of these differences, the conclusions were similar. Chen et al. (2011) and Lean and Rabiee (2011) reported, respectively, that the use of biotin supplements, generally at a dose of 20 mg day^{-1}, increases dry matter intake (0.87 and 0.70 kg day^{-1}), milk production

(1.66 and 1.27 kg day^{-1}), and increases or tends to increase milk fat yield (0.05 and 0.07 kg day^{-1}) and milk protein yield (0.05 and 0.02 kg day^{-1}). The vitamin supplement has no effect on milk fat and protein concentrations.

Folic acid

Folic acid is a cofactor for the transfer of one-carbon units obtained from serine, glycine, histidine, or formate catabolism. The vitamin is essential for DNA synthesis and replication, and thus, cell division. Folate metabolism also provides one-carbon units for *de novo* formation of methyl groups needed in numerous reactions, among them DNA methylation, which controls gene transcription and genetic stability, and synthesis of phosphatidylcholine, choline, creatine, and neurotransmitters, such as serotonin, dopamine, norepinephrin, and epinephrine (Choi and Mason, 2000).

Supplements of folic acid are frequently reported to increase milk production and milk protein concentration and yield in multiparous cows, but to have limited effect when given to primiparous cows (Girard et al., 1995; Girard and Matte, 1998; Li et al., 2016). Milk casein yield was increased and non-protein nitrogen content was decreased during the first 100 days of lactation in milk of multiparous cows fed supplementary folic acid without effect on dry matter intake, giving an indication that nitrogen was used more efficiently (Girard and Matte, 1998). However, in another study, Girard et al. (2005) observed no effect of daily dietary supplements of folic acid on lactation performance of multiparous dairy cows.

Vitamin B_{12}

Vitamin B_{12} acts as coenzyme in only two metabolic reactions. One of these vitamin B_{12}-dependent enzyme, methylmalonyl-coenzyme A mutase, plays a major role for the entry of propionate in the Krebs cycle and gluconeogenesis (McDowell, 2000). Besides this role, the vitamin is a coenzyme for the methionine synthase, the critical interface between folic acid and vitamin B_{12} metabolism. In summary, a vitamin B_{12} deficiency diverts all available one-carbon units towards the synthesis of 5-methyl-tetrahydrofolate, a form of folates that can be demethylated only by the methionine synthase, consequently leading to a secondary folate deficiency (Scott, 1999).

Supplements of vitamin B_{12} generally fail to affect milk and milk component yields in cows (Frobish et Davis, 1977; Croom et al., 1981; Akins et al., 2013; Graulet et al., 2017). However, Elliot et al. (1979) reported that bi-weekly intramuscular injections of vitamin B_{12} given from week 2 to 8 postpartum increased milk fat yield.

Cobalt is an essential component of vitamin B_{12}. Only bacteria and archaebacteria can synthesize the vitamin if cobalt supply is sufficient (Martens et al., 2002), which explains why cobalt dietary supplements are routinely used in dairy cow diets. Incidentally, it has been observed that, in spite of a dietary supply in cobalt equal to or greater than 0.11 mg per kilogram of dry matter ingested (NRC, 2001), serum vitamin B_{12} was frequently low in early lactation (Girard and Matte, 1999; Girard et al., 2005). Given the close interrelationship between folic acid and vitamin B_{12} metabolism, these observations suggest that a suboptimal vitamin B_{12} supply, especially during early lactation, may limit the effects of folic acid supplements. To verify this hypothesis, a study was conducted during the first weeks of lactation on primiparous cows fed a daily dietary supplement of folic acid; they received weekly intramuscular injections of either saline or vitamin B_{12} (Girard and Matte, 2005). Supplementary vitamin B_{12} increased energy-corrected milk, packed cell volume, and blood haemoglobin and decreased serum methylmalonic acid concentrations. The effect on blood hemoglobin and packed cell volume suggests that low vitamin B_{12} supply interfered with folate metabolism because folic acid deficiency, through its role in DNA synthesis, affects blood red cell formation (Bills et al., 1992). Accumulation of methylmalonic acid in serum indicates that low vitamin B_{12} supply also affected the other vitamin B_{12}-dependent enzyme, methylmalonyl-CoA mutase, essential to propionate utilization. These findings supported the hypothesis that differences in vitamin B_{12} supply may explain discrepancy among studies looking at the effects of folic acid supplements.

Indeed, a combined supplement of folic acid and vitamin B_{12} has been reported to improve metabolic efficiency, especially energy metabolism (Graulet et al., 2007; Preynat et al., 2009; Duplessis et al., 2014a; Gagnon et al., 2015). Moreover, possibly through an improvement of the energy balance in early lactation, the combined supplement of vitamins changed the expression of genes involved in differentiation of ovarian follicles (Gagnon et al., 2015), increased the number of large follicles and the size of the dominant follicle (Ghaemialehashemi, 2013), and decreased the interval between calving and the first insemination (Duplessis et al., 2014b).

Results described above show that dairy cows could benefit from B-vitamin supplements. Nevertheless, production and metabolic responses of dairy cows to B-vitamin supplements are highly variable among studies, even when the studies are conducted under similar experimental design and conditions. Determination of B-vitamin adequate supply recommendations required repeatability among studies conducted under different management practices. The following section aims to describe the major challenges to overcome to be able to issue B-vitamin recommendations for dairy cows.

Determination of B-vitamin adequate supply for dairy cows: challenges to overcome

In humans and non-ruminants, estimation of the minimum requirement, i.e. the lowest intake to support normal function, is essential to define a dietary recommendation for a specific nutrient. The first step to quantify the minimum requirement for a vitamin is to identify a marker, often the activity of an enzyme or the vitamin concentration in a specific tissue, which will respond early to a lack of the studied vitamin. The second step will be to feed a basal diet deficient only in this vitamin and supplemented with increasing doses of this nutrient to obtain a dose-response curve for the chosen marker (Combs, 2012). Determination of B-vitamin recommendations for dairy cows will require a similar approach, although there are supplementary challenges to overcome.

Challenge 1: Determine B-vitamin supply

In non-ruminants, because the diet is the only source of B vitamins, estimation of the minimum requirement for B vitamins is relatively straightforward. In dairy cows, however, the amounts of B vitamins synthesized or degraded by microorganisms present in the rumen should also be taken into account.

Results illustrated why, in dairy cows, the amount of B vitamins ingested is not a reliable indicator of the amount of vitamins reaching the sites of absorption and available for the animal (Table 1). In this example, cows fed diets based on alfalfa silage harvested at two different maturity stages. Niacin intake, when cows fed the diet based on early-cut alfalfa silage, was more than twice greater than when cows fed the diet based on late-cut alfalfa, but degradation of the vitamin in the rumen was nearly 10 times greater for the former. Consequently, in spite of a greater niacin intake, the amount of niacin reaching the small intestine was 21% smaller for cows fed the diet based on early-cut silage (Castagnino, 2016).

Moreover, all B vitamins did not respond alike to dietary changes (Table 2). In this study, cows fed isoenergetic diets based on corn silage with a forage-to-concentrate ratio of 60:40, on a dry matter basis. Two carbohydrate source treatments were compared: high in starch from barley, corn, and wheat or high in fiber from soybean hulls and dehydrated beet pulp. The carbohydrate source had only limited effect on thiamin and niacin intakes, apparent ruminal syntheses, and duodenal flows. Folate intake was lower with the high-starch diet, but because this diet promoted folate synthesis in the rumen, the amounts of folates reaching the duodenum did not differ between treatments. On the one hand, intake and duodenal flow of riboflavin and vitamin B_6 were greater with the high-starch diet. On the other hand, apparent ruminal synthesis and duodenal flow of vitamin B_{12} were three times lower when cows were fed the high-starch diets than when fed the high-fibre diets (Beaudet et al., 2016). These results illustrate that changes in diet composition and their effects on ruminal fermentation pattern play a major role on the fate of B vitamins in rumen and the amounts available for the cow. However, it also

Table 1 - Intake, duodenal flow, and apparent ruminal synthesis of niacin[1] in response to diets based on alfalfa silage harvested and ensiled as early- or late-cut[2]

	Early cut	Late cut	SEM	P-value
Niacin (mg day^{-1})				
Intake	4,423	1,902	77.6	<0.001
Apparent ruminal synthesis	−3,191	−300	112.6	<0.001
Duodenal flow	1,268	1,602	117.6	0.006

[1] Sum of the molar concentrations of nicotinic acid and nicotinamide.
[2] Adapted from Castagnino (2016).
SEM - standard error of the mean.

Table 2 - Intake, duodenal flow, and apparent ruminal synthesis of thiamin, riboflavin, niacin, vitamin B_6, folates, and vitamin B_{12} of cows receiving high-starch or high-fiber concentrate[1]

	Starch	Fiber	SEM	P-value
Intake (mg day^{-1})				
Thiamin	51	34	2.4	<0.01
Riboflavin	1172	1050	25.8	0.01
Niacin	2139	2192	50.2	0.50
Vitamin B_6	202	161	4.0	<0.01
Folates	11	15	0.4	<0.01
Vitamin B_{12}	0.09	0.09	0.002	0.56
Apparent ruminal synthesis (mg day^{-1})				
Thiamin	−10	−3	7.9	0.56
Riboflavin	374	41	102.8	0.06
Niacin	−601	−844	98.1	0.14
Vitamin B_6	−133	−115	6.8	0.11
Folates	25	14	3.6	0.07
Vitamin B_{12}	3	9	0.7	<0.01
Duodenal flow (mg day^{-1})				
Thiamin	41	31	7.3	0.41
Riboflavin	1546	1091	92.4	0.01
Niacin	1538	1348	104.5	0.26
Vitamin B_6	69	46	4.9	0.01
Folates	36	30	3.7	0.25
Vitamin B_{12}	3	9	0.7	<0.01

[1] Adapted from Beaudet et al. (2016).
SEM - standard error of the mean.

highlights the fact that effects on one vitamin cannot be extrapolated to another.

Nevertheless, knowledge on the factors controlling the amounts of B vitamins escaping the rumen and then available for absorption by the dairy cow is limited. Therefore, the major challenge to face for research on B-vitamin requirements of dairy cows to progress is to be able to predict B-vitamin supply according to diet chemical composition. This step is crucial to establish relevant B-vitamin recommendations for dairy cows. In summary, the challenge is: How can we decide if a cow needs a B-vitamin supplement if we have no idea about the amounts reaching the sites of absorption in the small intestine?

Challenge 2: Identify specific biomarkers for each B vitamin

Most studies on dairy cows have used lactation performance as a marker to determine adequate B-vitamin supply. Nevertheless, this marker is non-specific and likely to be altered only at a later stage of deficiency. Identification of a more sensitive marker, specifically related to one of the metabolic functions of the studied vitamin, is needed. Identification of such biomarker could be speeded up by using knowledge from studies conducted in humans and non-ruminant animals. Nevertheless, the adequacy of the chosen biomarkers will need to be assessed in cows to take into account the specificity of their metabolism, for example, the major importance of gluconeogenesis as compared to non-ruminant animals.

Challenge 3: Analytical methods

This last challenge may look rather trivial. Nevertheless, measuring B vitamins in feedstuffs and duodenal or omasal digesta is also quite a challenge for the following reasons:

In feedstuffs and digesta, B vitamins are embedded in a complex matrix, mostly protein and starch. Extraction procedures using a mixture of enzymes, such as protease and amylase, should be standardized to ensure a complete liberation of the vitamins before quantification. Moreover, some B vitamins require specific treatments. For example, folates, which can be present as mono- or polyglutamates, require a treatment with conjugase, an enzyme which shorten the glutamate chain, before microbiological determination. Biotin is present in feedstuffs and duodenal digesta as biotin and biocytin. The latter is a molecule of biotin bound to protein lysyl residues by an amide link. This bond cannot be hydrolyzed by proteases; it can be broken only by the enzyme biotinidase. Vitamin B_{12} is present mostly as hydroxycobalamin, methylcobalamin, or adenosylcobalamin; once freed of their embedding matrix, these forms are rapidly destroyed by light and heat. Therefore, they should be rapidly transformed in a stable form, cyanocobalamin, by heating in presence of cyanide before analysis.

Most B vitamins are present in feedstuffs and digesta under more than one active form. For example, vitamin B_6 is present as pyridoxine, pyridoxamine, and pyridoxal as well as all the phosphorylated forms of these vitamers. Thus, the method used should detect all these forms or be able to transform them efficiently.

The last challenge, although not the least:concentrations of B vitamins in these media are frequently in nano- or picogramme per gramme of dry matter. Therefore, the analytical methods chosen need to be highly sensitive.

Conclusions

Research on B-vitamin requirements of dairy cows is still in its very early stage. In addition to the methodological problems for reliable vitamin determination, there is a whole new research field to be conquered. The number of published studies on production and metabolic responses of dairy cows to B-vitamin supplements is still very low and studies comparing effects of increasing doses of a vitamin are nearly non-existent. Production and metabolic responses of dairy cows to B-vitamin supplements are highly variable among studies. Although quantification of the metabolic demand for B vitamins is still far from precise, such variability among studies is likely, partially due to differences in the amounts of B vitamins available for the cow. The major challenge to overcome presently is being able to predict B-vitamin supply. If the basal supply is adequate, a positive effect of a B-vitamin supplement is unlikely, whereas a positive response to supplementation can be expected if the supply is sub-optimal. Consequently, B-vitamin adequate intake recommendations for dairy cows will vary according to their total supply, i.e. the amounts of vitamins from dietary sources escaping degradation in the rumen and the amounts of vitamins synthesized in the rumen. Prediction of the fate of B vitamins in the rumen according to diet chemical composition is therefore essential to establish such recommendations.

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