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College of Agriculture and Life Sciences
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Viewing a Role for Isoacids in Dairy Nutrition Through a New Lens

J.L. Firkins
Department of Animal Sciences
The Ohio State University

Introduction

The main branched-chain volatile fatty acids (BCVFA) in the rumen include isovaleric (IV), 2-methylbutyric (2MB), and isobutyric (IB) acids, which are produced from the branched chain amino acids (BCAA) leucine, isoleucine, and valine, respectively. They were previously included with valerate, which is a straight-chain VFA, in a calcium salt in a product, IsoPlus™. This product was marketed in the mid to late 1980's by Eastman Kodak. That company supported numerous studies with rumen inoculum in vitro, digestion trials, and especially lactation trials. They evaluated different concentrations of BCVFA under differing dietary conditions before seemingly coming to a general formula. From the inception of our research collaborations on what became IsoFerm® with Zinpro (Eden Prairie, MN), we have focused on mechanism to look through the new lens regarding which BCVFA are most responsive mechanistically to help determine which dietary conditions those BCVFA would be useful additives for today's dairy cows.

Valerate was included in IsoPlus based on numerous other inclusions in studies on BCVFA. Valerate is derived from several amino acids (AA) or produced during fermentation of glucose (primarily from starch). Even the bacteria that were shown to require valerate probably could substitute some other carbon sources such as acetate, which would be plentiful in the rumen (but not in the culture), so valerate probably never needed to be included (Roman-Garcia et al., 2021a). Because of the critical need for BCVFA by cellulolytics, we deemed neutral detergent fiber degradability (NDFD) as the main response criterion in these in vitro studies. After ruling out valerate, we documented that the BCVFA all improved NDFD but were most useful in the order of 2-methylbutyrate, isobutyrate, and isovalerate. Because leucine is high in corn protein and because isobutyrate could substitute for isovalerate in bacterial pure cultures, we hypothesized that isovalerate might not need to be supplemented.

Foundational microbiology studies have established that the BCVFA are converted to BCAA for protein synthesis in bacteria and archaea (methanogens) that have lost one or more key enzymes needed to synthesize the BCAA (Figure 1). Unlike most characterized bacteria (such as enteric bacteria), the majority of ruminal bacteria appear to use some sort of ferredoxin-dependent branched chain keto acid dehydrogenase (or similar enzyme) to produce the BCVFA-CoA. This enzyme complex is important because it is much more reversible than the characterized enzyme. It probably also explains why inhibition of methanogenesis prevented BCVFA formation (Hino and Russell, 1985). The Stickland reaction in clostridia, probably those known as hyper-ammonia producers, has an unknown importance in deamination of BCAA.

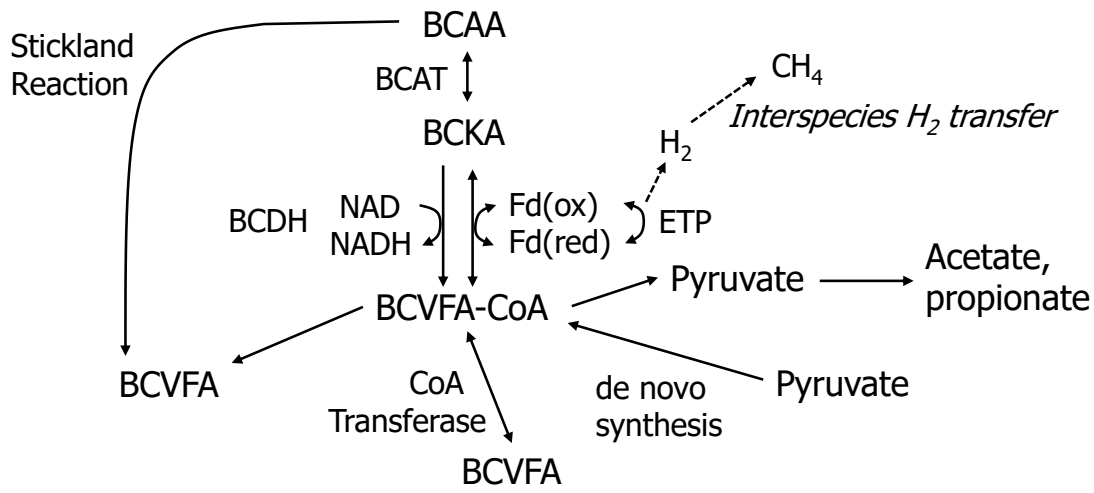

Figure 1. Likely mechanism by which isoacids are used by ruminal bacteria. BCAA = branched chain amino acids, BCVFA = branched chain VFA, BCKA = branched chain keto acid, BCAT = branched chain aminotransferase, BCDH = branched chain keto acid dehydrogenase, Fd = ferredoxin that is oxidized or reduced, and ETP = electron transport phosphorylation to make ATP.

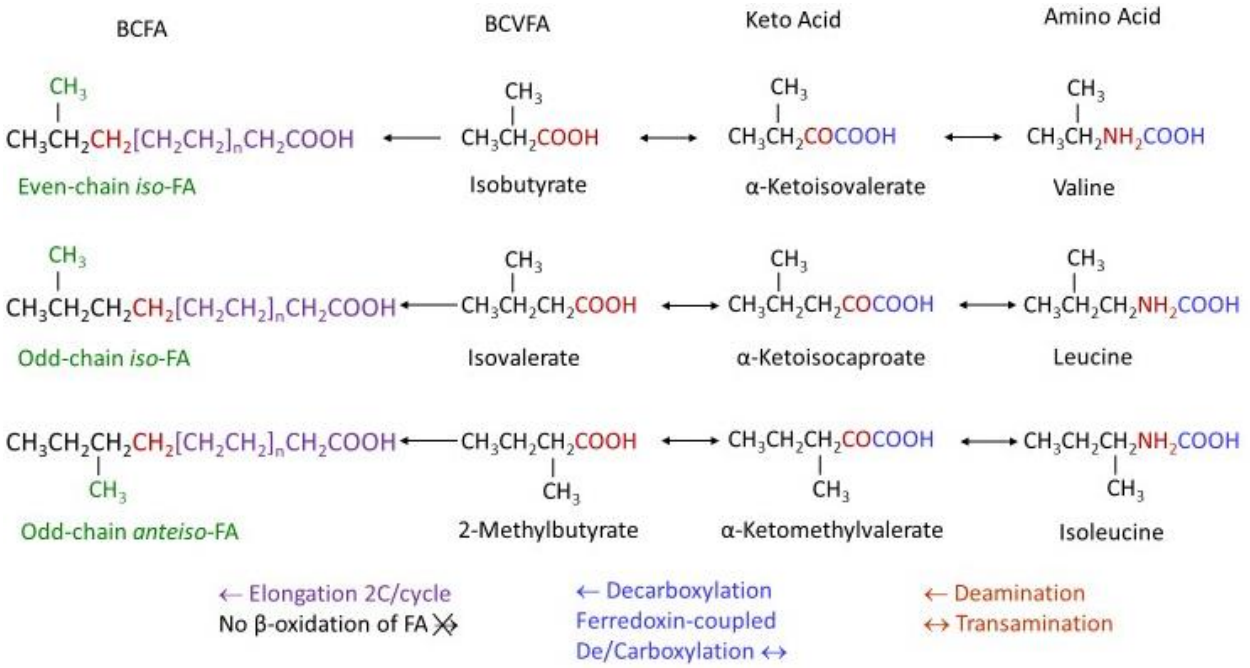

Figure 2 Unlike with aerobic cells, β-oxidation of FA is unlikely to shorten these chains in the anaerobic rumen. Anteiso configurations are on the opposite side of the BCFA than iso methyl groups and are more fluidizing.

The BCFA are primarily located in phospholipids in rumen bacteria (or protozoa that engulf them). Unlike aerobic bacteria, once fatty acids (FA) are attached to the phospholipids, there is no desaturation (which requires oxygen) combined with saturation to control membrane fluidity in anaerobic bacteria. This is different than

biohydrogenation of free unsaturated FA that occurs on the exterior of the bacterial membrane and uses reducing intracellular reducing equivalents. The classical representation of membranes includes a bilayer of phospholipids. The bilayers cannot be packed too densely or too fluid to maintain membrane integrity. Rather than the kink caused by a double bond from unsaturated FA, anaerobes either take in oleic acid from the rumen fluid or build their own BCFA from BCVFA primers (Figure 2), with the methyl group spreading the FA apart on the phospholipid. The anteiso-BCFA makes the membrane more fluid than iso-BCFA. Thus, anaerobic bacteria regulate the BCFA:straight chain FA in their membranes to maintain its integrity. Combinations of longer or shorter chains and iso or anteiso branches combine for varying conditions. Elongation of BCVFA to BCFA is conserved in anaerobic bacteria.

The landmark papers of Dr. Milt Allison from the Agricultural Research Service documented these responses for BCAA or BCFA synthesis for various pure cultures of bacteria from the 1960's to the 1980's; although not verified, they were repeatedly ahead of their time in suggesting the role of ferredoxin in conversion of BCVFA to BCAA (and therefore reversed to make BCVFA; Figure 1) and also suggestive of an important role for branched chain aldehydes (discussed herein) to aid cellulolytic bacteria. Much of this work led to the studies supporting IsoPlus but also were the springboard for our research. The objective of this paper is to explain the role of BCVFA in microbial function and to adapt those mechanisms to dairy cattle nutrition. Any interested reader can contact me for details and references.

Role for BCVFA for Fiber Degradation

Many major reviews have documented the BCVFA requirements for cellulolytic *Fibrobacter* and *Ruminococcus* species of ruminal bacteria both in historical terms (Andries et al., 1987) and more current applications (Firkins, 2021; Firkins and Mitchell, 2023). These cellulolytics have often been determined to be in the "core" group that are found nearly universally and therefore have an inferred critical function for ruminants, including dairy cattle. That these core bacteria can thrive even though they lack the ability to produce some critical precursors such as the BCAA supports 1) they are specialists (i.e., unique and important) and 2) that they cross-feed synergistically with other microbes that produce those precursors while benefiting from the interaction. Moraïs and Mizrahi (2019) describe these dynamic interrelationships for the BCVFA producers and cellulolytic users. They also documented studies in which *Treponema* chemotactically seek forage even though they do not degrade fiber (some use pectin). Their corkscrew shape and rotation help pull the nonmotile cellulolytics into feed particles. *Treponema* is among other non-cellulolytics that require one or more BCVFA and work synergistically with cellulolytics (Roman-Garcia et al., 2021b). That referenced study and another from our lab (Mitchell et al., 2023d) document that the BCVFA-requiring *Ruminococcus bromii* probably competes with cellulolytics for BCVFA in mixed diets if BCVFA supply is low. When rumen-degraded protein (RDP) was limited to dairy cattle, the relative abundance of cellulolytic bacteria was decreased, and their lowest abundance coincided with the time after feeding (2.5 hours) when the BCVFA were in lowest concentration (Belanche et al., 2012).

The major characterized cellulolytics also contribute to hemicellulose degradation even if they do not use the resultant sugars for fermentation; however, cellulolytics have poor ability to degrade RDP. Hemicellulolytic and pectinolytic bacteria help to degrade protein to AA, BCVFA, and ammonia needed by cellulolytics, but the main proteolytics probably are amylolytic bacteria. Some of these proteolytics that produce and do not require BCVFA still can use BCVFA for BCAA synthesis if RDP is limited. These sorts of competitive interactions for key nutritional resources can unbalance the microbial consortium and must be one important reason why adding starch to dairy diets still decreases ruminal NDFD (Ferraretto et al., 2013). Diluting starch with byproduct NDF helps to diminish this depressed NDFD, but providing adequate RDP to provide BCVFA also likely helps limit these negative associative effects (Firkins, 2010).

Several studies have documented the complex repertoire of enzymes and associated molecules that provide function to those enzymes (Gruninger et al., 2019; Moraïs and Mizrahi, 2019). After initial attachment, cellulolytics degrade polymers of cellulose and hemicellulose, and use the resultant oligosaccharides or sugars in metabolism. These proteins are manufactured inside the cell (at the ribosome) and then processed and moved through the cell membrane to be assembled and anchored on the exterior of the cell. Many details of these functions resulting from adhesion to fiber, including extracellular assembly of enzymes, were charted (Miron et al., 2001) prior to filling in gaps based on meta-genomics (Naas and Pope, 2020). The membrane must be flexible enough to allow this translocation while remaining rigid enough to serve as a barrier. Gram-positive bacteria have a thicker cell wall with carbohydrate, protein, and lipid components but only one membrane. Gram-negative bacteria have two membranes, with a periplasmic space between them. Despite these differences in cell walls types, bacteria requiring BCVFA cross multiple cell wall types and phyla.

We have known for decades that rumen bacteria lipids that were chemically measured to contain both straight- and branched-chain FA and aldehydes. Prior to genomics, the lipid profile was associated with different groups of bacteria. Research went dark for several decades after streamlining of hydrolysis and methylation steps prior to FA analysis such that these aldehydes now are eluting or coeluting with FA in standard chromatography approaches. Combining isotopic labeling with an improved approach derived from the literature allowed us to “go back to the future” to separate, identify, and quantify the aldehydes (Mitchell et al., 2023a). In that study, even though aldehydes were only about 6% of the total lipids, about 26% of the recovered ^{13}C-labeled BCVFA was in aldehydes because about 50% of the aldehydes were branched-chain. We discussed how FA on the first carbon of glycerol can be converted to a vinyl ether in a plasmalogen and subsequently measured as an aldehyde.

Plasmalogens are well known in cell biology because of important roles in human health. However, much less is known in anaerobes. They are widespread across various phyla of anaerobes and yet are documented in only a few cases of aerobes. As explained and cited by Mitchell et al. (2023a), they appear to provide two key functions: 1) physical stability of the membrane and 2) oxygen scavenging capacity. We do not

know why branched FA are prioritized for the reaction that creates plasmalogens, but the methyl branch might counteract the vinyl ether to prevent over-rigid membranes. Although not well studied, plasmalogen concentrations probably depend on growth conditions. Our work suggests that FA elongated from 2-methylbutyrate have a constant and important role in both FA and plasmalogens in phospholipids, those from isovalerate have a lesser role regardless of conditions (more is converted to its parent AA, leucine), and those plasmalogens formed from isobutyrate appeared to be the most responsive to changing culture conditions. Our work clearly showed a much higher (43%) recovery of ^{13}C-BCVFA when we increased the forage:concentrate, documenting the importance of branched lipids for fibrolytic bacteria.

This goldilocks fluidity of membranes is very important in rumen bacteria as they compete for substrate under differing ruminal pH, unsaturated fat availability, etc. BCFA would be replaced by straight-chain FA to prevent over-fluidizing of the membrane if bacteria are taking up oleic acid from supplemental fat. In contrast to our hypothesis that adding unsaturated fat would increase their uptake into membranes and decrease the need for BCFA, 2MB was not decreased as a primer elongating to anteiso FA under differing conditions affected by forage:concentrate, extra unsaturated fat, or decreasing pH. In nonrumen bacteria, these anteiso FA are known to be prioritized for the 2nd carbon of glycerol in the phospholipid. If so for rumen bacteria, the importance of the vinyl ether only on the first glycerol carbon of a plasmalogen needs further research to explain. Regardless, the important role for branched lipids in the BCVFA-requiring cellulolytics also ripples through the associated cross-feeders that require BCVFA (such as *Treponema*) or those that use BCVFA but do not require them (Roman-Garcia et al., 2021b; Mitchell et al., 2023d).

The second and emerging role for plasmalogens appears to protect bacteria from oxygen. Some retrospective studies associated an inverse relationship between redox (more negative value means less oxygen) and NDFD (Mitchell et al., 2023d). Microbial additives appear to improve NDFD at least partly through oxygen scavenging (Firkins and Mitchell, 2023). More and more rumen bacteria are producing energy through nontraditional pathways, typically involving formation of ion gradients through protein complexes spanning the membrane. The key cofactor for electron bifurcation (the secret sauce for energy conservation) in these protein complexes is a cofactor that also is its “Achilles heel” (as described by one author we cited) in the presence of molecular oxygen. The double bond of plasmalogens in membranes could be protecting these membrane-spanning protein complexes from oxygen in the feed or water so that bacteria can attach, colonize, and degrade ingested feed more effectively.

Rapid colonization is critical as the cellulolytics interact with the noncellulolytics to jockey for position; once position is established, the race for degradation extends until the increasingly colonized particle passes from the rumen. Paul Weimer at the USDA suggested a principle for this ordered progression benefits from prevention of low pH for establishment of cellulolytics initially after a new feeding. I have elaborated on this concept with respect to sharing nutrient resources (Firkins, 2010). The BCVFA and ammonia concentrations are not necessarily congruent during this process because

protein breakdown to AA is done primarily by amylolytics that funnel those AA and ammonia into their cellular proteins rather than sharing with cellulolytics. Thus, the different waves of bacterial types during colonization of plant matter (Gruninger et al., 2019; Moraïs and Mizrahi, 2019) can become limited for fiber degradation.

Why is all of this discussion critical for lactating dairy cattle? Decreased NDFD is a loss of energy, of course, but less NDFD also could contribute to increased rumen fill and decreased dry matter intake (DMI) in dairy cattle. Many of the studies from Mike Allen while he was a faculty member at Michigan State University documented that animals with increasing “demand” for energy are greater responders to improvement in ruminal NDFD resulting from better quality forage, for example. This effect is further complicated by forage fragility; more fragile forages are easier to break down in size and pass from the rumen to alleviate fill (Allen et al., 2019). Many field nutritionists are using undegraded NDF (uNDF) in ration formulation and are well aware of these issues. My point is that, if we want to optimize the ruminal digestibility of potentially degradable fiber, key nutrients such as adequate nitrogen or BCVFA must be available for the fibrolytic microbes in the rumen, and these conditions might not necessarily be replicated in typical uNDF assays. Presumably, these same issues apply for fibrous byproducts as for forages.

Feeding BCVFA increased fiber digestibility as assessed by rumen fibrolytic enzyme assays and total tract NDFD, and these results corresponded with increased relative abundance of BCVFA-requiring cellulolytics in dairy cattle (Wang et al., 2019). Several similar responses were shown in beef cattle. Some studies were dose responses that documented a plateau at an intermediate dose. However, these studies typically did not limit RDP or even attempt to measure or discuss it, which will become important in subsequent discussion.

The Role for BCVFA for Efficiency of Microbial Protein Synthesis

Peter Van Soest’s classic book (Nutritional Ecology of the Ruminant) and other papers of that vintage catalogued general trends for increased microbial protein production but decreased efficiency of microbial protein synthesis (EMPS) with increased concentrate in the diet. However, these observations were done before today’s expectations for meta-analyses and even before starch was typically measured. In the first CNCPS, Russell et al. (1992) established a higher maintenance energy coefficient for bacteria degrading nonstructural carbohydrate (NSC) compared with bacteria degrading structural carbohydrate (SC). A greater proportion of energy for maintenance would correspond with a decreased EMPS because a greater proportion of energy is expended in maintenance. They also predicted the role of peptides for these NSC degraders. Subsequent Cornell work has made other improvements that are beyond my scope. However, peptides were not predicted to influence the SC bacteria, presumably because if peptides were sufficient for the NSC bacteria, then there were presumably adequate peptide precursors for BCVFA needed by SC bacteria. There has never been any suggestion that BCVFA are not important, just not limiting.

The concentration of added BCVFA that was deemed not limiting for fibrolytic microbes in batch culture (Gorosito et al., 1985) was far below the BCVFA concentrations measured in dairy cattle; in fact, those authors did not actually measure the total BCVFA concentration. Although I also often rely on batch culture for research, these in vitro concentrations should not be extrapolated to in vivo conditions. Logically, a ~ 4-fold dilution by buffer (0.5-gram substrate in 25 mL of fluid yields about 2% DM in batch cultures), whereas rumen contents are 8 to 10% DM. If we account for that dilution, a 0.3 mM addition of BCVFA would be 1.2 to 1.5 mM in vivo, which puts their responsiveness in the same range associated with their need to improve efficiency of microbial protein synthesis from dairy cattle. Although Gorosito et al. (1985) documented some important points such as lack of valerate effect and the BCVFA can substitute for each other, they also collected fluid-associated bacteria as their inoculum, which was prior to numerous studies documenting the need to extract particulate-phase bacteria to optimize NDFD. The adherent bacteria outnumber the fluid-phase bacteria by about 3:1, and the highly efficient cellulolytics are highly adherent. Roman-Garcia et al. (2021a) also diluted rumen fluid by 4 fold but used inoculum that was enriched in the particulate-phase bacteria, noting consistent improvement in NDFD when BCVFA were added, also ranking the BCVFA in the order of 2MB > IB > IV.

Russell et al. (1983) and many other authors have documented that peptide breakdown is the rate-limiting step for conversion of RDP into peptides that are further catabolized to small peptides, AA, or ammonia to be assimilated into microbial protein. A potential role for increasing isoacids to limit peptide breakdown (see later discussion) could be why their supplementation of peptides was somewhat better than provision of BCVFA alone for cellulose and hemicellulose degradation (Russell et al., 1983).

Soluble protein passes out of the rumen and might even be underestimated by the NASEM (2021) model compared with other models such as CNCPS. Although not well documented, this soluble protein could contain a lot of peptides. Some sort of end-product inhibition of peptide breakdown has been repeatedly hypothesized but not documented (Mitchell et al., 2023b). In pure cultures of characterized proteolytics, most (but not all) strains generally agree with the mixed batch culture results and with important description of their particular enzymes (Walker et al., 2005). Less clear is the supposition that hydrophobic peptides are catabolized less than hydrophilic ones, but, if so, peptides with more BCAA might be degraded less than peptides with less BCAA. Of the preformed AA that are taken up by bacteria, phenylalanine, leucine, and isoleucine stand out (Atasoglu et al., 2004; Bach et al., 2005).

Continuous culture offers some advantages for studying nitrogen metabolism by ruminal microbes (although protozoal numbers are lower). We can measure total flows without flow markers and total VFA production without isotopes. Mitchell et al. (2023b) noted that adding a mix of all three BCVFA increased microbial protein flow and EMPS both by about 7%. However, surprisingly, the total flow (bacterial plus nonbacterial) AA was increased by over 10% for all AA except tryptophan. The BCAA increased mostly because of bacterial BCAA flow, whereas proline and alanine total flows increased by 17 and 21%, respectively. Peptides with these AA are known to be hydrolysis sites for

dipeptidyl peptidases, which probably contribute most to peptide breakdown in the rumen. One or more BCVFA can increase the RUP of protein as estimated in situ, although responses are not always consistent (Table 1). Increased 2MB could lead to increased intracellular isoleucine imbalance relative to the other BCAA for ruminal bacteria (Kajikawa et al., 2005), but it is important to note that the protease assay and RUP derived from in situ measurements likely did not account for peptide accumulation. Mitchell et al. (2023b) tried to account for this disparity by theorizing that increased intracellular concentration of BCAA (especially leucine from IV) after dosing BCVFA provide an important transcriptional queue for AA adequacy by proteolytic bacteria to prioritize AA for anabolic processes (i.e., growth) vs AA for peptidases and other catabolic responses.

Table 1. Studies measuring ruminal protein degradation after feeding isoacids.

BCVFA	Protease	RUP (in situ)	Urease	Study
Isovalerate	↓ Linearly	↓ Linearly	↓ Linearly	Liu et al. (2014)
Isobutyrate	↓ Linearly	↓ Linearly	↓ Linearly	Wang et al. (2015)
2-Methybutyrate	↓ Linearly	↓ Linearly	↓ Linearly	Zhang et al. (2015)
2-Methylbutyrate	↑	↑		Wang et al. (2018a)
2-Methylbutyrate	↑	↑		Wang et al. (2018b)
All BCVFA	↑			Wang et al. (2019)

The first five studies were with steers, and the sixth was with lactating dairy cows.

Bacteria are very high in protein, and the BCAA comprise nearly 20% of the true protein. Thus, cells seem to sense AA adequacy (typically one or all of the BCAA) and try to integrate that with ammonia (probably through glutamate) and carbon adequacy (from degraded carbohydrate). If N and AA are limiting, proteolytic bacteria upregulate peptidases to provide AA and a little bit of energy from AA degradation for themselves; if AA are in excess, downregulating peptidases would save all of the AA used to build the number and variety of peptidases needed to yield those AA. Expression of proteases appears more constitutive (constant) because proteases are needed to break through the protein matrix surrounding starch granules in grain.

Emerging research suggests that the isoacids are not going to be as effective if we limit overall N to microbes. The proteolytic bacteria producing AA and ammonia use those products for themselves rather than releasing AA and ammonia for usage by the nonproteolytic bacteria largely responsible for NDFD. Ideally, isoacids would partially substitute for RDP and allow a lower protein cost. However, the more biologically correct interpretation appears to be that isoacids can help stimulate EMPS to convert RDP more efficiently into microbial protein and decrease the wastage of RDP. A deficiency of nitrogenous growth factors for cellulolytics could depress NDFD and DMI. Greater NDFD should increase the amount of microbial protein but also an improvement in EMPS probably based on a more balanced consortium but also potentially because cellulolytics have lower maintenance coefficients, as parameterized in CNCPS. When isoacids improved both NDFD and EMPS in continuous culture, there was no important shift in bacterial population (Mitchell et al., 2023d). Similarly, isoacids helped prevent a decreased diversity of bacteria resulting from a higher grain diet (Lee et al., 2021).

Peptides or nitrogen have long been known to be important to minimize non-growth expenditure of energy (Hackmann and Firkins, 2015), which is especially important for lactating dairy cattle fed highly fermentable diets at high feed intakes.

Nutritionists are trying to formulate diets for RDP to meet microbial protein, but there likely is no RDP requirement. NASEM (2021) capped RDP at 12%, but increased RDP without a change in rumen-degraded carbohydrate should increase the growth of bacteria that can use AA for fuel. For example, NRC (2001) expected RDP to be converted to microbial protein at 85%. When using extra RDP to increase microbial protein in the NASEM (2021) model, that efficiency probably drops to 50% or lower. Hanigan et al. (2021), expanding efforts to investigate RDP, concluded that the RDP effect on EMPS was stubbornly linear. With respect to isoacid supplementation, the response surface from our meta-analysis (Roman-Garcia et al., 2016) begins to make more sense if it is restricted to ammonia concentrations more realistic for dairy cattle not overfed protein (Figure 3). Although these are not cause and effect regressions, responses in Figure 3 support an increasing benefit from isoacids with adequate ammonia. Unfortunately, “isovalerate” is reported in about 90% of papers that exclude 2MB because it coelutes with (and usually a higher concentration than) IV. Readers are urged to read the report by Lapierre and Van Amburgh in this proceedings for important updates on why rumen nitrogen must be met to reach the potential of isoacids.

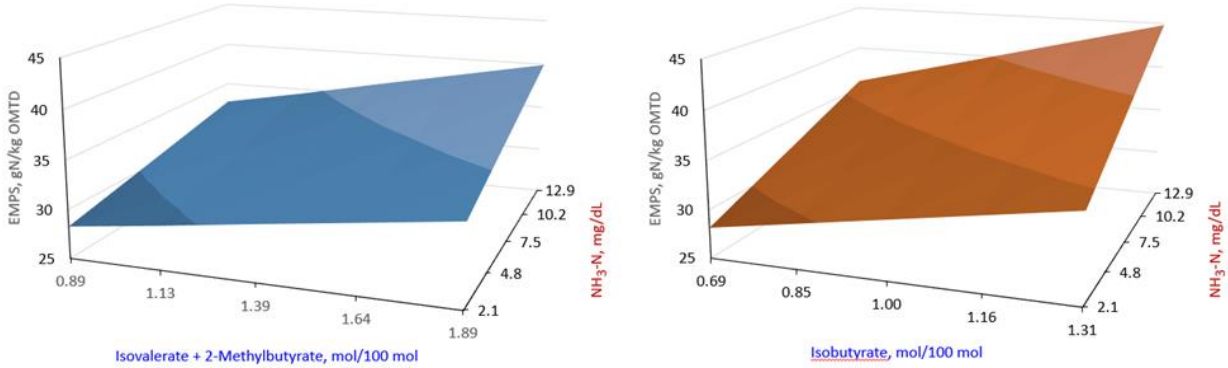

Figure 3. The equations from Roman-Garcia et al. (2016) to predict efficiency of microbial protein synthesis (EMPS; Y axis) were truncated to the mean ammonia concentration of 12.9 mg/dL projecting downward (Z axis) and shown for increasing isovalerate (actually a mixture of IV and 2MB) or isobutyrate on the X axis.

Production R**esponses from BCVFA**

The older IsoPlus papers from the 1980’s often had papers with multiple studies and over longer lengths of time than many of the more current studies. Andries et al. (1987) reviewed the literature and noted milk production improved from 4 to 13% in different studies compared with their controls. Increased microbial protein production was a proposed mechanism for increased growth hormone concentration and its role in milk production. More current research also has supported a potential increase in

growth hormone (Wang et al., 2019). A subsequent evaluation of different doses from four university studies noted a 5.3 and 10.7% increase in milk production (and protein production) for cows in mid and late lactation when fed ammonium salts of BCVFA at 0.8% of the concentrate (Otterby et al., 1990); no response was noted for early lactation. The product was 74% BCVFA, which were 31.0, 25.2, 19.4, and 24.4% IB, 2MB, IV, and valerate. They suggested that the early lactation diets had enough soybean meal as the main protein source to limit benefits of BCVFA. They enrolled only multiparous cows, and they cited lower responses from primiparous cows in previous studies. In a simple (no statistics) summary of 54 field studies, supplementing isoacids increased milk production over time from 0.64 to 1.91 kg/d for the first through third month on trial (McBride, 1987). Seven of those studies had negative milk production responses, and one could speculate that these might have been limiting in RDP.

All of these IsoPlus studies were with dairy cows with much lower DMI and milk production potential compared with today, whereas certain trends also were noted in the more current studies. Multiparous cows responded more to isoacids in our study (Mitchell et al., 2023c). In that study, we noted that 2MB was not effective without IB. We reasoned that the high corn in our diet should provide enough IV without supplementation, which was generally supported by the data. I am not inferring that heifers will not respond; they just are more likely to show their response through body weight gain. An improvement in feed efficiency of about 7.5% was detected for IB + 2MB compared with control in our study. However, when all the isoacids were supplemented in another study, milk production improved by about 2.5 kg/d (~8%) in multiparous cows starting in the third month of lactation (Wang et al., 2019). NDFD increased by about 4% units, and the authors noted increased growth hormone concentration (as discussed above). In another study from the same group in China (Liu et al., 2018a), multiparous cows again were enrolled in their third month of lactation. The milk response to BCVFA was linear, but the middle amount of 60 g/d of the three BCVFA increased milk (7.4%) and milkfat (14.9%) production with little numerical improvement thereafter. Mammary gland biopsies revealed BCVFA increased expression of transcription factors (PPARγ and SREBP) and genes needed for de novo synthesis of FA. Increased concentration of the short and medium chain FA associated with mammary de novo synthesis in their study corresponded with a similar increase in these de novo FA in our study (Lee et al., 2021). In dairy calves fed BCVFA, increased liver PPARα expression was associated with increased liver oxidation of long chain FA (Liu et al., 2018b). Beyond my scope here, some of this group's studies documented improved rumen epithelium development in calves. The straight-chain VFA are well known for increasing expression of PPAR; perhaps one or more BCVFA also have a role in transcription if they get past the liver. However, because increased acetate supply increases mammary synthesis of FA (Matamoros et al., 2022), we assume improved NDFD and increased acetate production is important to achieving the full benefit of isoacid supplementation.

In nearly all of these studies, the dietary CP was at least 16.0%, whereas our unpublished work documents a lack of response to BCVFA in a low (slightly < 15.0%) CP diet in Holstein cows (White et al., 2023) compared with higher CP (a mixture of

more RDP and RUP). In that study, residuals analyses suggested a key relationship in which those cows with milk urea N (MUN) below 8 mg/dL responded poorly or negatively to BCVFA supplementation, whereas those above 8 mg/dL responded positively. April White's PhD unpublished research at Ohio State supports also shows inverse relationships between ruminal ammonia N and responsiveness to BCVFA using these low protein diets, suggesting the MUN response in the production study reflected ruminal N deficiency. We also noted no benefit to isoacids unless RDP was increased from 9 to 11% in another preliminary analysis (Park et al., 2023). Our interpretation is that the dosed BCVFA are less assimilated into microbial matter and more prone to passage or absorption from the rumen. The fed BCVFA therefore should increase the concentration of BCVFA in the rumen to increase the intracellular concentration, assimilating more in BCAA or BCFA. When ammonia was kept above limiting concentrations in continuous culture, Mitchell et al. (2023b) documented increased recovery of BCVFA in BCAA and BCFA with higher forage, presumably because of the higher NDF and higher NDFD.

Isovalerate probably is metabolized by rumen epithelium, whereas IB is virtually unmetabolized (Kristensen et al., 2000). This stands to reason because, according to textbooks on BCAA oxidation, IV (after esterification with coenzyme A) yields acetoacetate and acetyl-CoA, whereas IB yields propionyl-CoA. In our unpublished study, increasing IB increased propionate molar percentage, perhaps indicating some rumen microbial metabolism. The rumen epithelium is well known to prioritize butyrate as fuel (and its pathway can include acetoacetyl-CoA). To my knowledge, such work with 2MB has not been done (it should yield one each of acetyl-CoA and propionyl-CoA). In contrast with the rumen tissue, isobutyrate and 2-methylbutyrate appear to be metabolized more by the liver more than is isovalerate (Reynolds et al., 1988). Thus, we would expect relatively little of the absorbed BCVFA dose to get past the rumen (IV) and liver (IB and 2MB). Thus, most authors have suggested that improved NDFD indirectly improves rumen development and milk FA synthesis because of greater production of butyrate and acetate, respectively.

As with when milk fat depression from feeding unsaturated fat diverts the dietary fat to the adipose tissue, if there is any postruminal response from BCVFA that does not stimulate milk FA synthesis, we might expect some partitioning toward the cows' body tissues. In our studies, we have noted such inverse responses between milk fat and body weight gain. This response might be more pronounced with animals in first lactation that are still growing. Thus, a rumen deficiency of nitrogen should especially avoided for those animals. Interestingly, preliminary work from Jackie Boerman's lab at Purdue suggests a role for BCVFA to decrease BW loss in transition cows (Gouveia et al., 2023) and influence fetal muscle activity (Gast et al., 2023), suggesting partitioning to the fetus and then to protect against BW loss after calving. Further work is needed, but clearly isoacid supplementation must be considered beyond milk production.

Conclusions

After looking at isoacids through a new lens, the picture is emerging of a more complex relationship than just providing growth factors for cellulolytics. Clearly, we want to start by protection against blurring of cellulolytics' BCVFA and nitrogen needs, particularly when there is competition by amylolytics. Increasing BCVFA concentration in rumen fluid of supplemented cows should increase BCVFA concentration inside bacteria, which should increase BCAA concentration. Intracellular BCAA probably trigger global transcription of a variety of genes in bacteria, including those used for growth relative to those processes supporting that growth. Awaiting verification is our hypothesis that BCVFA can decrease peptide breakdown and ammonia production. Hence, rumen nitrogen must be adequate for isoacids to work effectively. Although there are potential direct effects of any BCVFA that are absorbed, an improved fiber digestibility could also lead to increased FA synthesis in the mammary gland through supplying more acetate but also through transcriptional regulation. Improved energy availability probably helps to increase milk production without drawing on body reserves. Because primiparous cows are still growing, they might respond less to isoacids for milk production but could partition more energy to growth. Improved NDFD should either allow similar milk production with a slightly lower DMI in mid to late lactation or increased DMI and higher milk production in early lactation (probably past the first ~ 4 weeks for those cows with high demand. Our results suggest that isovalerate is needed less than isobutyrate, but if dairy diets have lower leucine resulting from lower corn protein, isovalerate might be needed. Finally, I can attest that reformulating isoacids away from ammonium salts provides a more palatable product, but nutritionists should be careful to make sure that cows do not sort against the product.

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Taking the Stink Out of Branched Chain VFAs
Capturing Targeted Nutrient Feeding through Modeling

P. Andrew LaPierre and M.E. Van Amburgh
Department of Animal Science
Cornell University

Introduction

What was once old, is new again. And perhaps what's the best thing about this freshening up, is the lack of odor associated with it. Having never personally experienced the odor that kept many farm families at odds with each other, testimonials are still rather vivid whenever the word 'IsoPlus' is whispered in a dairy industry crowd. Previously developed in the 1980s as a byproduct of film making, the FDA had approved IsoPlus as a feed additive for dairy cattle diets. Claims of 'four to six pounds more milk per cow per day with minimal changes to their diet' can still be found with a quick YouTube search. The product, however, was short lived on the market (One could imagine the kitchen table conversations involving IsoPlus), yet the biological relevance of branched chain VFAs (BCVFA) is foundational when discussing the proper functionality of the rumen microbiome. Recent product development has blown the dust off this technology, taking a fresh approach to masking the odor surrounding these volatile compounds all while maintaining their mode of action. Obviously, dietary formulation has come a long way over the last thirty years, but with ever-increasing pressures for balancing nutrient supplies to boost productive efficiencies and minimize excessive nutrient excretion, a case for feeding exogenous isoacids can still be made. Most dietary formulation model, unfortunately, would not appropriately predict the effects isoacid feeding might have within the rumen environment, particularly in circumstances where they would be warranted. This paper aims to make a case where updates could be made to the Cornell Net Carbohydrate and Protein System (CNCPS) to better represent the effects of meeting this requirement would do to fiber digestibility and productive efficiency.

Where do BCVFA fit in rumen metabolism?

It has been long known that cellulolytic bacteria, including *Fibrobacter succinogenes, Ruminococcus albus,* and *Ruminococcus flavefaciens,* exhibit requirements for BCVFA (Andries et al., 1987) with the intention of using them to synthesize branch chain amino acids (BCAA) through reductive carboxylation (Allison et al., 1962b, Allison, 1969, Robinson and Allison, 1969) or branch chain fatty acids (BCFA) through chain elongation (Allison et al., 1962a). These cellulolytic bacteria lack the ability to r intercellularly transport or synthesize one or more BCAA necessary for growth and proliferation (Mitchell et al., 2023a). The majority of BCVFA found in the rumen are by-products of BCAA metabolism from other dominant species of bacteria, whereby, the deamination of the amino group or transamination of the corresponding keto acid occurs. Ironically, it has been observed that the amylolytic bacteria, *Prevotella ruminicola,* which does not require BCVFA to synthesize BCAA, will preferentially carboxylate them back to

BCAA, down regulating de novo BCAA production and reducing cross feeding to bacteria who cannot synthesize BCAA without BCVFA as the precursor (Allison et al., 1984). A strong argument can be made that the reduction in aNDFom digestibility observed in dairy cattle with higher levels of dietary starch (de Souza et al., 2018) is due to this resource competition. Bacteria that digest starch have the capacity to take up peptides and amino acids (Russell and Sniffen, 1984; Chen et al., 1987) which provides them a competitive advantage over fiber digesting bacteria which can only utilize ammonia and have a much slower growth rate (Bryant, 1973). Exogenous supplementation of these BCVFA has shown improved aNDFom disappearance in batch cultures (Russell and Sniffen, 1984, Cummins and Papas, 1985, Roman-Garcia et al., 2021a), providing necessary growth factors for primary colonizers in fibrotic material; however, in diets where true rumen degraded protein (RDP less ammonia concentration) is provided in sufficient amounts, no such improvements in disappearance are observed (Copelin et al., 2021). Further, supplying these iso-acids has demonstrated improvements in dry matter intake, milk volume (Andries et al., 1987), and milk components (Wang et al., 2019).

Where do BCVFA fit in diet formulation modeling?

Under the current structure of CNCPS v6.5.5 (Fox et al., 2004, Tylutki et al., 2008, Van Amburgh et al., 2015) predictions for the ruminal disappearance of carbohydrate and proteins adhere to first order kinetics which are calculated using rates of degradation and passage for each ingredient in percent per hour, establishing the maximum potential with which a particular feed fraction can be degraded. Additionally, a microbial yield coefficient is also calculated for each carbohydrate fraction in the diet, using the intrinsic rate of degradation for each feed ingredient in percent per hour, the maintenance rate of bacteria in grams of CHO per gram of bacteria, and growth potential of that bacteria in grams of bacteria per gram of CHO. Each yield coefficient is applied to the corresponding feed fraction to estimate microbial yield for a given feed. Summation of the microbial yields for CHO A2, A3, A4, B1, and B2 feed fractions represents the potential non-fiber carbohydrate (NFC) degrading bacteria pool whereas CHO B3 is the sole carbohydrate fraction used to estimate the potential fiber carbohydrate (FC) degrading bacteria pool size (Fox et al., 2004). These estimates of carbohydrate disappearance and microbial pool size are predicated on the assumption that the rumen is not limited in other growth factors, especially nitrogenous substrates. The CNCPS estimates total available rumen N by summating dietary ammonia intake (PRO A1), recycled N (Recktenwald et al., 2014), and dietary peptides and degraded feed N from microbial action. This N is pooled together to estimate the potential bacterial growth based on the N content of both NFC and FC degrading bacteria (Russell et al., 1992). This pool is then appropriated to each ingredient within the diet based on the proportion that each feed ingredient contributes to the total degraded carbohydrate (i.e., if feed ingredient one contributed 15% of the degrade carbohydrate from the diet, then 15% of the total nitrogen pool is appropriated to the degradation of that ingredient). Taken with the carbohydrate allowable microbial yields, the system considers whether the amount of N appropriated to each feed ingredient is sufficient to achieve the potential carbohydrate degradation calculated. If the predicted allowable N pool is inadequate to complement the potential carbohydrate degradation, the system will restrict the level of carbohydrate degraded, prioritizing a

limitation of fiber carbohydrate degradation first, based on metabolic activities of NFC and FC bacteria. Further, this limitation in available N and degraded carbohydrates will depress predicted bacterial flow, causing a reduction in metabolizable protein (MP) which would hinder productivity of the animal.

As the industry looks to reduce protein feeding in lactating diets, the likelihood that a nutritionist would encounter a scenario where predicted rumen N is not sufficient to meet potential carbohydrate degradation is greater than previously seen. When such dietary scenarios present themselves, it is imperative to recognize certain limitations under the current CNCPS predictions. Presently, the CNCPS amalgamates all nitrogenous substrates into one N pool, considering them all equal when reconciling the necessary substrates for proper microbial metabolism, proliferation, and feed degradation. As such, in instances where nitrogen supply is low, inclusion of dietary urea may be used as a method to improve rumen total N pool size, giving the appearance in the model that sufficient N is present to realize potential carbohydrate degradation and microbial yield. This solution creates a fallacy, as rumen ammonia supply is likely not the prevailing cause for this limitation in carbohydrate degradation. Branch chain amino acids, which are currently not considered in the rumen N pool, but, because they are precursors for BCVFA, also have a unique carbon backbone used for other metabolic processes, become limited in scenarios when dietary protein is concomitantly reduced. This presents an opportunity to disaggregate the rumen N pool, allowing for the consideration of BCAA/BCVFA sufficiency.

To account for BCAA/BCVFA adequacy in the CNCPS, an approach similar to Tedeschi et al. (2000) has been integrated into the CNCPS and its preliminary results are shown below. Like the predicted supply of other nutrients, the user defined feed chemistry of dietary ingredients will be used to estimate the supply of BCAA and any exogenous sources of BCVFA. Because oxidative deamination of BCAA by microbes is still the primary source of ruminal BCVFA (Allison et al., 1962b), the rate of BCAA deamination, release of BCVFA by bacteria, and assimilation of BCVFA by other bacteria are all important considerations in predicting the status of BCVFA within the rumen. At present, work from Atasoglu et al. (2004) is being used to estimate the rate in which BCAA are oxidatively deaminated to BCVFA by amylolytic bacteria and the proportion of excreted BCVFA which are taken up by fibrolytic bacteria. The primary fates of BCVFA are defined in the CNCPS as BCAA or BCFA, with the understanding that a small proportion of BCVFA may be used for branch chain keto acid production (Firkins, 2021). Bacterial protein content and amino acid profile data already exists within the CNCPS and is used to predict the amount of bacterial outflow from the rumen (Russell et al., 1992); however, only the bacterial fat content and not the profile of fatty acids, and further BCFA, is expressed in the CNCPS. As such, fatty acid profile data from Vlaeminck et al. (2006) has been used to profile the fatty acid composition of bacteria and identifying which of these fatty acids are classified as BCFA to establish recommended feeding rates. Taking all these predictions together, the supply of BCVFA from the conditions specified by the user are used to estimate the allowable bacterial growth from BCAA/BCVFA supply. Similar to the concept of the total rumen N pool, in situations where rumen degraded BCAA and exogenous BCVFA are not sufficient enough to meet the potential microbial

growth from degraded carbohydrates, the system will restrict carbohydrate degradation and subsequent microbial growth based on what is supplied. Independent of the rumen N pool, a user of the CNCPS would be able to troubleshoot whether a low protein diet was limited in either rumen N, BCAA/BCVFA, or a combination of both. As such, limitation born from BCVFA deficiencies cannot be overcome with the supplementation of urea and will only be reconciled when either true RDP with an appropriate level of BCAA or a concomitant substitution of exogenous BCVFA are provided.

To test this approach, a data set consisting of 1,352 treatment means (Table 1) ranging in true RDP, starch, and fiber content were evaluated using the amended version of the CNCPS. This evaluation identified which of these diets had no substrate limitation when degrading the potentially degradable carbohydrate substrate from the diet, while flagging which of them were limited by either rumen N or by the BCAA/BCVFA. These nutrients (BCAA/BCVFA) are being used synonymously because limited data exists to identify whether the limitation lies with BCAA or BCVFA as one can be made from the other and they are cycled extensively (Firkins, 2021). Of the diets which were flagged for a BCAA/BCVFA limitation, an exogenous source of BCVFA was included in each of the diets at a rate which would overcome this limitation. The diets were rerun through the CNCPS, and the results collected. The primary responses that the CNCPS predicted was improvements to aNDFom digestibility (NDFD), MP from bacterial sources, and potential milk yield (Table 2). Multiple experiments conducted in continuous cultures indicated a 5.0% (Roman-Garcia et al., 2021b) and 7.6% (Mitchell et al., 2023b) improvement in NDFD when BCVFA were supplemented under various dietary conditions. Average improvements from this evaluation suggested a 3.7% increase in NDFD, complementing the results observed in the literature. Consequently, the improvements in NDFD resulted in an increase in MP from bacteria, averaging 32.1 g, and a concomitant increase in predicted milk of approximately1.1 kg.

Responses to the supplementation of exogenous BCVFA in the revised CNCPS are graphically depicted in Figure 1, 2 and 3. Each figure displays all diets which were either limited in N, limited in BCAA/BCVFA, or showed no limitation when evaluating the potentially degradable carbohydrate from the diet. The data in Figure 1 describes the predicted differences when comparing this response to the diets starch and potentially digestible (pd) aNDFom content, whereas Figures 2 and 3 show the describes similar differences when related to either starch and true RDP or fiber content and true RDP content, respectively. Not surprisingly, nearly all diets with a higher NDF content expressed a demand for BCVFA supplementation. Of these diets, those with greater starch content (>35% DM) showed an even greater increase in NDFD; however, nearly all dairy cattle are below this level of starch content. True RDP content was less of an influence on the demand for BCVFA when pdNDFom and starch content was considered, as diets across the entire range of true RDP were considered limited in BCVFA, particularly when pdNDFom content was high. It is worth noting that there were several higher fiber diets which were not flagged as being limited in BCVFA because the AA profile of the ingredients within the diet had a larger portion of BCAA available for rumen metabolism.

Table 1. Descriptive statistics for dataset used to evaluate BCVFA updates within CNCPS.

Parameter	n	Mean	Std	Min	Max
Dry Matter Intake	1325	18.7	4.1	6.9	30.4
Original Milk Production, kg		27.8	7.5	4.8	48.2
True RDP, % DM		9.9	1.6	5.2	19.5
Starch, % DM		24.3	9.8	0.6	56.4
aNDFom, % DM		33.6	6.9	14.8	62.6
pdNDFom, % DM		25.0	6.3	10.0	51.2

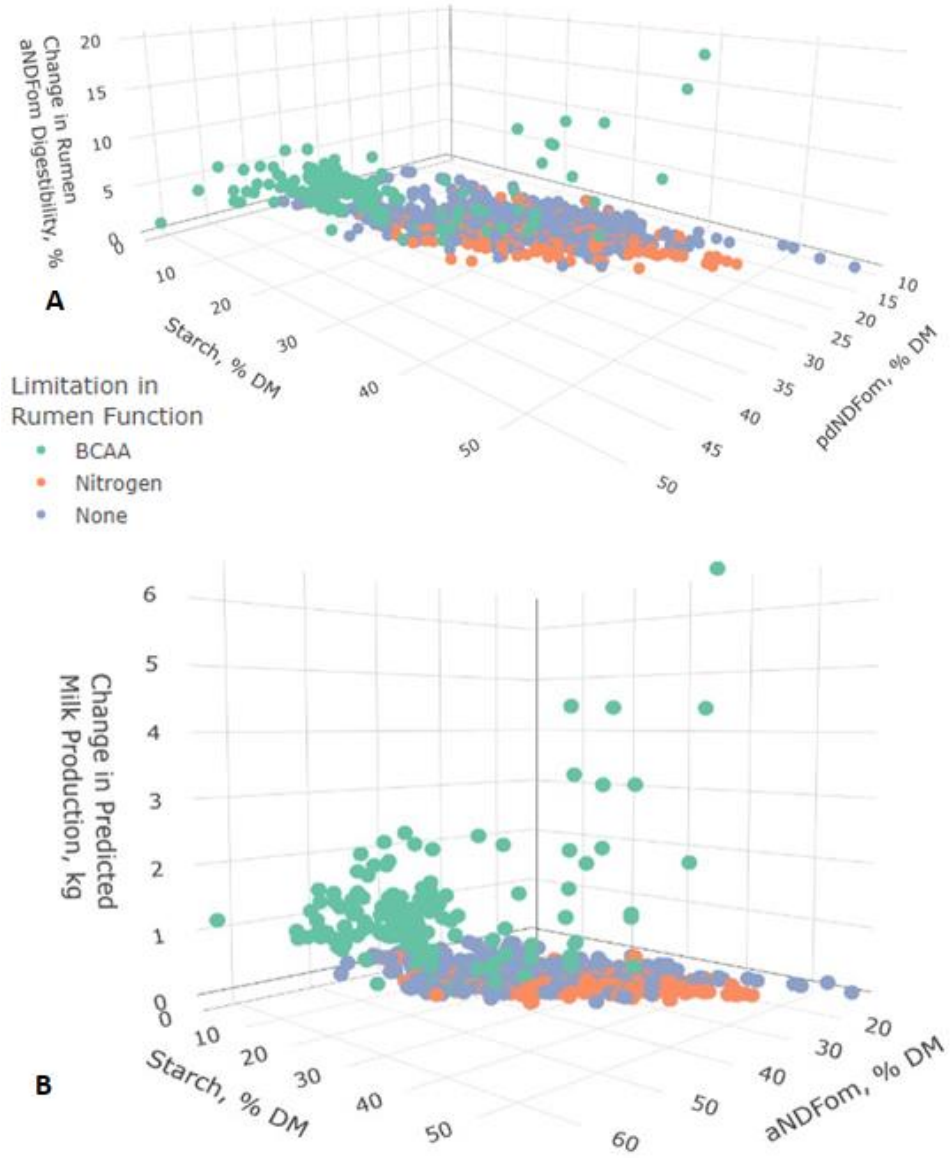

Figure 1A & B. Relationship between dietary starch content, fiber content, and changes in either predicted rumen fiber degradation or milk production with the addition of isoacids to diets which are either limited by N supply, BCAA supply, or are not limited by nitrogenous supply.

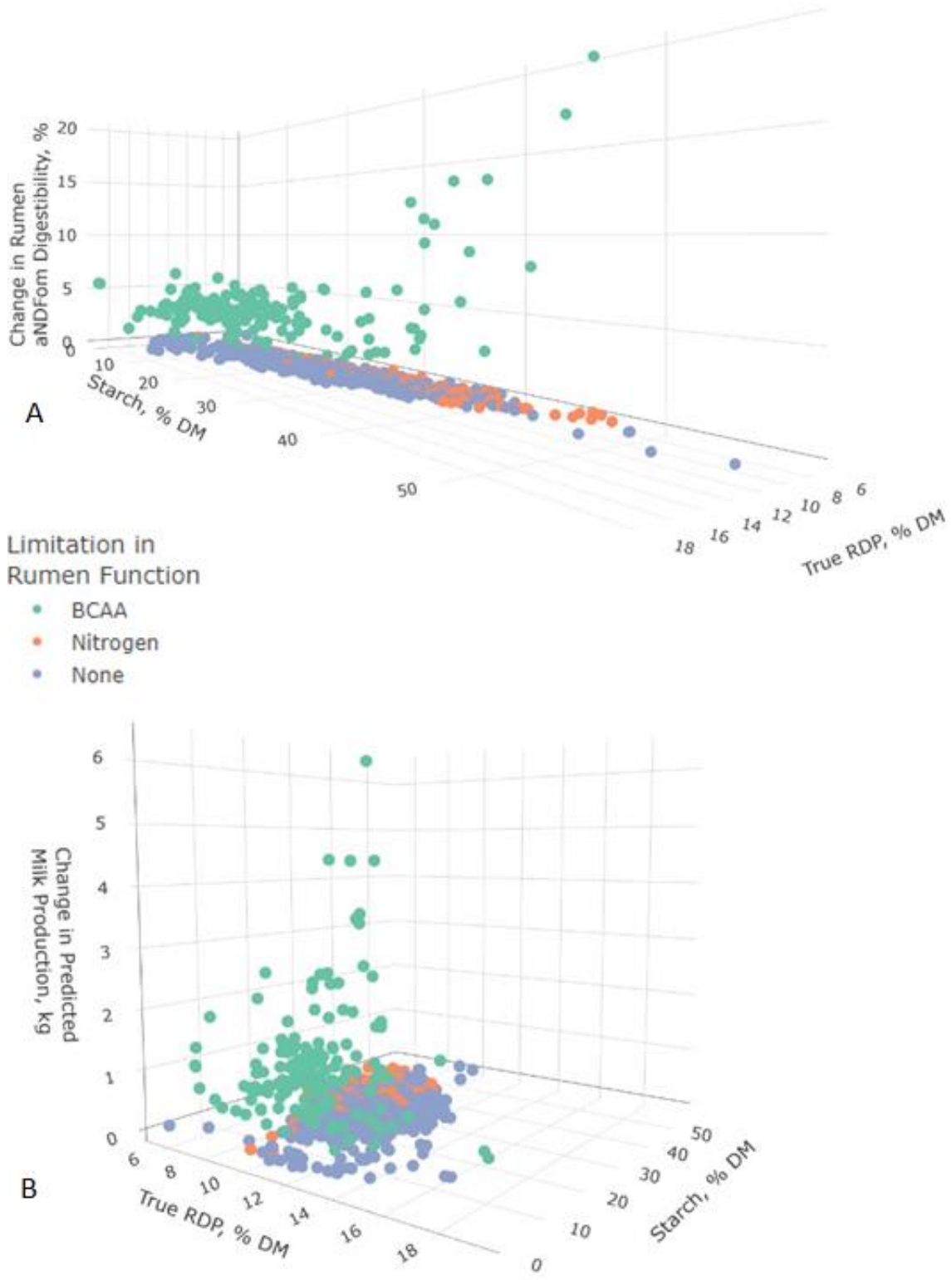

Figure 2A & B. Relationship between dietary starch content, true RDP content, and changes in either predicted rumen fiber degradation or milk production with the addition of isoacids to diets which are either limited by N supply, BCAA supply, or are not limited by nitrogenous supply.

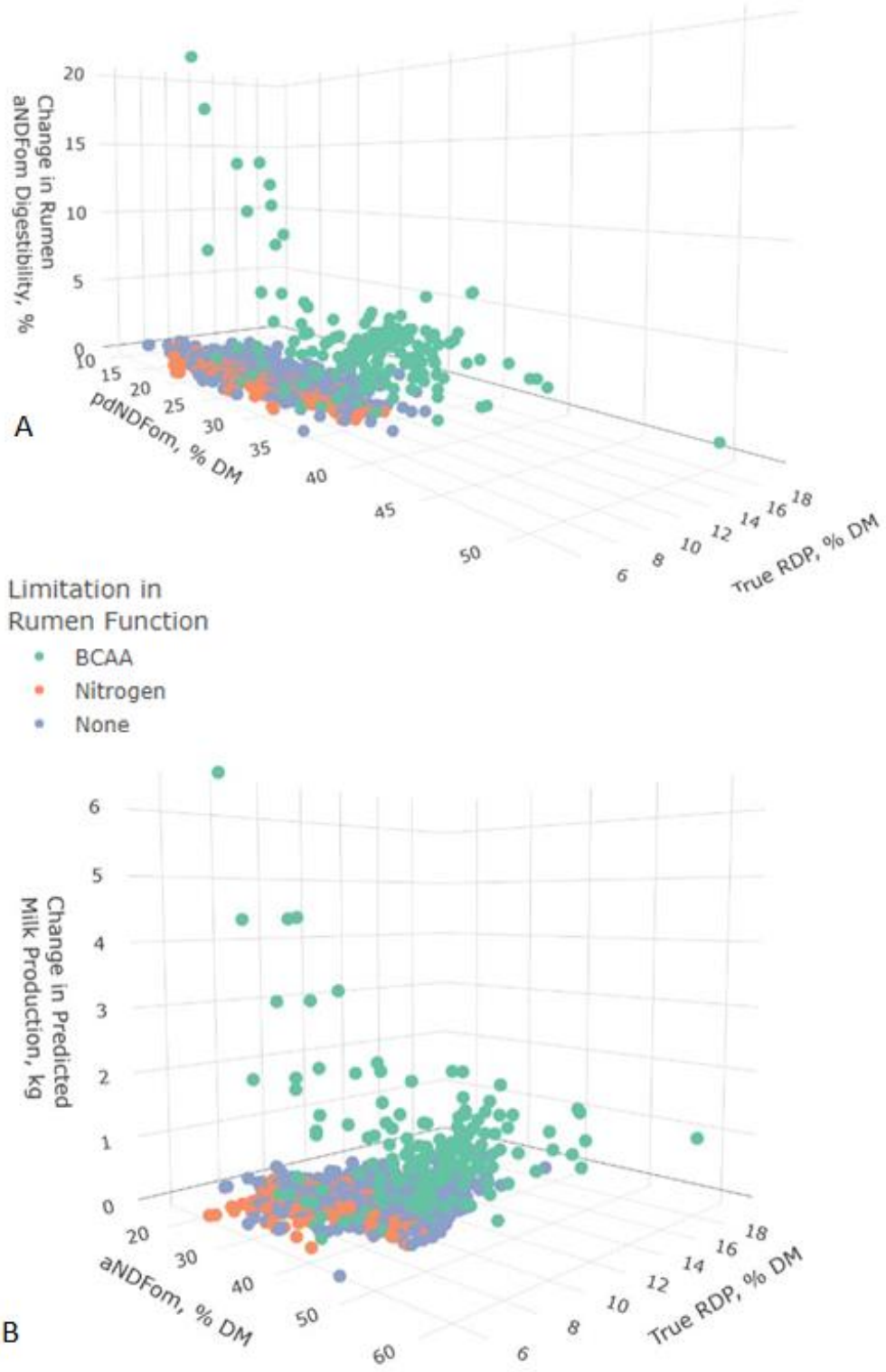

Figure 3A & B. Relationship between dietary fiber content, true RDP content, and changes in either predicted rumen fiber degradation or milk production with the addition of isoacids to diets which are either limited by N supply, BCAA supply, or are not limited by nitrogenous supply.

Table 2. Average predicted response and relationship to dietary aNDFom, starch and true rumen degradable protein for rumen aNDFom degradability, metabolizable protein sourced from bacteria, and milk production. Responses are a result of revisions to the CNCPS to better represent BCVFA supplementation.

		Regression Analysis				
Response to BCVFA Supplementation	**Average Response**	**R-Squared**	**Parameter**	**Estimate**	**Std Error**	***P*-Value**
Rumen aNDFom degradability, %	3.7	0.44	Intercept	6.46	2.852	0.02
			aNDFom	-0.098	0.0475	0.04
			Starch	0.092	0.0300	0.00
			True RDP	0.0248	0.0958	0.80
Bacterial MP, g	32.1	0.41	Intercept	-0.329	0.7987	0.68
			aNDFom	0.0216	0.0169	0.20
			Starch	0.0439	0.0089	< 0.01
			True RDP	0.0081	0.0299	0.79
Milk yield, kg	1.10	0.41	Intercept	0.440	0.9129	0.63
			aNDFom	0.0025	0.0152	0.87
			Starch	0.038	0.0096	0.00
			True RDP	0.0028	0.0307	0.93

Conclusions

The improvements discussed here serve as the first step toward a continued evolution to predicted more discrete nutrient interactions within the rumen that have real implications on fiber degradation, microbial yield, and cattle performance. As more research works to describe microbial metabolisms surrounding BCVFA, there will be efforts made to improve not only the predictions but also the structure surrounding the predictions, so that there will more discrete outputs from the CNCPS which will aid in diagnostic work surrounding lower protein diets and which nutrients may be hindering optimal digestibility and microbial efficiency.

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Odd and Branched-Chain Fatty Acid Metabolism: Food Abundance and Human Physiology

J. Thomas Brenna
Departments of Pediatrics, Chemistry, and Nutrition, University of Texas at Austin
and
Division of Nutritional Sciences, Cornell University

Introduction

Most fatty acids in mammals are referred to as “straight chain” because their carbon chains are linear; further, they normally have even numbers of carbon atoms (Figure 1). Odd and branched chain fatty acids (OBCFA) are biosynthesized by rumen bacteria and appear in the milk and meat of ruminant animals. Though intakes of OBCFA are natural for humans they are seldom studied.

Dairy products are the major sources of odd and branched chain fatty acids OBCFA) in the human diets in North America [1]. U.S. cow’s milkfat contains about 2%BCFA distributed across seven main fatty acids (Table 1). The mean human dietary BCFA intakes in the US exceed 500 mg/d compared to less than 100 mg/d for the well-studied omega-3 long chain polyunsaturated fatty acids. BCFA are present in human milk at levels lower than in cow’s milk. Our objective is to review the body of recent evidence indicating that BCFA are key underconsumed nutrients for human gut health.

Table 1. Distribution of branched chain fatty acids in U.S. retail milk [2]

BCFA	FA (%, w/w)	BCFA (%)
iso-14:0	0.13 ± 0.04	6.4 ± 1.4
iso-15:0	0.13 ± 0.01	6.6 ± 0.4
anteiso-15:0	0.56 ± 0.03	27.5 ± 1.3
iso-16:0	0.31 ± 0.03	14.9 ± 0.9
iso-17:0	0.26 ± 0.02	12.7 ± 0.7
anteiso-17:0	0.61 ± 0.06	29.9 ± 2.0
iso-18:0	0.04 ± 0.02	1.9 ± 0.9
Total	2.04%	

Effects of OBCFA in Human Nutrition

When human fetal or adult-like enterocytes are treated with BCFA as free fatty acids, they are taken up and rapidly incorporated into membrane phospholipids to 30%-60% depending on structure. When administered in place of linoleic acid rich oils, they reduce the incidence of necrotizing enterocolitis in a neonatal rat model of the disease. In so doing, they increase the inflammatory cytokine IL-10 and shift the nascent microbiota toward organisms that contain high levels of BCFA in their membranes.

Occurrence in human nutrition

We established that BCFA are major components of the first solid meal of the prenatal (fetal) humans via oral intake of amniotic fluid borne vernix caseosa particles, where BCFA averages 30%. BCFA in California sea lion vernix develops mid-way through gestation, where they are found in vernix, stomach contents, amniotic fluid, plasma and meconium of fetal sea lions, all paralleling humans.

Occurrence in Yak milk

Among ruminant milks, milks of yaks (Bos grunniens or Poephagus grunniens) are richest in BCFA, especially in the “half-lactating” yak where BCFA concentrations average 5.3%. Yak manure is particularly rich in BCFA, averaging almost 15% BCFA. Peoples of the Qinghai-Tibetan plateau rely on yak milk and dairy products as staples, consuming at the extremes 3,500-5,000 mg of BCFA per day.

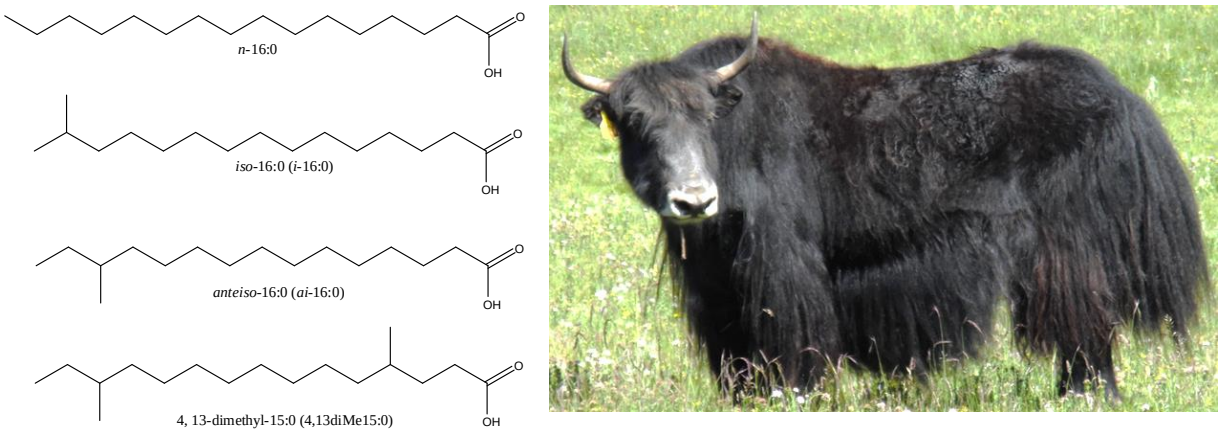

Figure 1. Chemical structures of straight chain (“normal”) fatty acids and branched chain fatty acids (BCFA). Right. Tibetan Yak 牦牛 (máoniú) milk has the highest known level of BCFA.

Summary

Odd and branched chain fatty acids abundant in diets of dairy consumers. They are bioactive but their nutritional properties are not well studied. Greater knowledge of these natural nutrients is likely to lead to more consumer awareness and drive demand for dairy.

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Potential of Plant-derived Bioactives and Polyphenols to Abate Enteric Methane Emission and Heat Stress in Ruminants

F. Ahmadi[1], H. A. R. Suleria[1], B. Tunkala[1], P. Prathap[1], S. S. Chauhan[1], F. R. Dunshea[1,2]
[1]School of Agriculture, Food and Ecosystem Sciences, Faculty of Science, The University of Melbourne, Australia
[2]Faculty of Biological Sciences, The University of Leeds, UK

Introduction

Climate change has emerged as a critical concern at the global level, impacting animal welfare and productivity, especially in tropical areas. Methane (CH_4), a potent greenhouse gas (GHG), is primarily generated by ruminant animals within the livestock sector. Owing to the high global warming potential of CH_4, it is crucial to identify the most effective and applicable strategies to abate CH_4 emissions from ruminant livestock. The ruminant livestock sector both fuels global warming and suffers from its consequences. Heat stress (HS) is a climate factor that impairs livestock productivity and health, causing financial losses for producers as well as environmental concerns due to increased CH_4 emission intensity, a measure of GHG emitted per unit of product (e.g., milk or meat). A consequence of HS is oxidative stress, which has been shown to be at least partially offset by supranutritional supplementation with antioxidants. However, the level of antioxidants required is higher than allowed in many jurisdictions, so alternatives are being sought. This mini-review aims to discuss the potential of plant-derived bioactive compounds in mitigating CH_4 emission from enteric fermentation and alleviating the negative effects of HS in ruminant livestock; the frequency of the latter is expected to intensify according to the climate projections (IPCC, 2022). Feeding high-concentrate diets is also briefly discussed as a potential strategy in the context of CH_4 emission and HS abatement and how plant bioactives could modulate the rumen fermentation perturbations.

Plant Bioactives

Bioactive compounds derived from plant secondary metabolism have been extensively researched in ruminant livestock, mainly as ruminal flora modifiers. Plant bioactives are categorized as Generally Recognized as Safe by the Food and Drug Administration and have strong public acceptance. A large body of literature supports the efficacy of these compounds, most notably tannins, saponins and other polyphenols, in mitigating CH_4 production from enteric fermentation in ruminants. This reduction is achieved mainly via a direct effect on methanogenic archaea, disruption of protozoa membranes, or shifting rumen fermentation toward more propionate production rather than methanogenesis (Wang et al., 2023). Another potential mechanism is via increasing the amount of protein and starch that resists rumen fermentation while still maintaining whole tract digestibility.

The positive effects of plant bioactives on animal health and productivity are well documented (Rochfort et al., 2008). Recent findings also support their positive effects on ruminant livestock under HS. Exposure to HS impairs health and productivity of ruminants (Thornton et al., 2022), increasing the carbon footprint of the production system. Literature evidence suggests that some plant-derived metabolites could attenuate the devastating effect of HS on livestock productivity and health and thus reduce CH_4 emissions intensity from enteric fermentation.

Enteric CH_4 Emission

The CH_4 mitigation strategies offer a mutually beneficial solution as reducing CH_4 emission redirects dietary energy loss (8–12%) from CH_4 towards productive purposes while reducing the anthropogenic contribution of ruminant animals to GHG emissions (Perry et al., 2016). It is outside this review's scope to discuss the potential options recommended to mitigate enteric CH_4 emissions in ruminants. For an in-depth review of the currently available strategies for enteric CH_4 mitigation, readers are invited to refer to recent review papers (Beauchemin et al., 2022; Hristov et al., 2022).

Condensed tannins (CT) have effectively mitigated enteric CH_4 production, mainly through their direct inhibitory effect on methanogens. *Desmanthus*, a tropical legume, is a rich source of CT and its inclusion at 31% of dry matter intake in beef cattle diet translated to a 10% decrease in CH_4 yield (Suybeng et al., 2020). In grazing beef systems, forages rich in CT have also been suggested as a potential CH_4-mitigation practice (Thompson and Rowntree, 2020). In the Australian grazing system, 10–20% CH_4 emission reductions and improved productivity have been reported in cattle browsing *Desmanthus* and *Leucaena* species as rich sources of CT (Black et al., 2021). A recent meta-analysis (Arndt et al., 2022) found that *Sericea lespedeza* is a promising tanniferous forage, reducing daily CH_4 emissions by 32% without harming feed intake, a potential concern with tannin-rich forages in ruminant diets (Rochfort et al., 2008). Feeding 400 g/d of tannin from *Acacia mearnsii* (600 g/kg CT) in combination with cottonseed oil (800 g/day) synergistically mitigated enteric CH_4 production in dairy cows by 20% (Williams et al., 2020). In addition to their antimethanogenic effect, diets rich in CT have exhibited antinematodal properties in ruminants (Rochfort et al., 2008).

Undigested CT excreted into manure may restart their CH_4-inhibiting activity by limiting methanogenic activity occurring in the manure, thereby contributing to the overall mitigation of GHG emissions (Lazzari et al., 2023). Moreover, adding CT to the ruminant diet has been suggested to lower manure-derived nitrous oxide (N_2O) emissions. N_2O is a potent GHG and one of the main contributors (up to 6%) to the global warming budget (Shakoor et al., 2021). Tannins bind to dietary proteins and improve N utilization in ruminant livestock. Shifting N excretion from urine to feces decreases N_2O emissions from the manure (Hristov et al., 2022). The protein–CT complex excreted into the manure is resistant to degradation in the soil. This increased recalcitrance potentially reduces N_2O emissions (Eckard et al., 2010). Providing steers with *S. lespedeza* effectively lowered GHG emissions (CO_2, CH_4, and N_2O) from manure deposited in the soil (van Cleef et al., 2022).

Animals grazing in the tropics and subtropics, characterized by pastures deficient in N are particularly vulnerable to dietary tannins (Waghorn, 2008). The potential costs and adverse effects on feed intake (increased astringency) and nutrient digestibility, particularly in high-yielding animals, also constrain their use in ruminant nutrition (Beauchemin et al., 2022). Microbial adaptation within the rumen ecosystem is another concern that may limit the long-term effectiveness of this mitigation strategy.

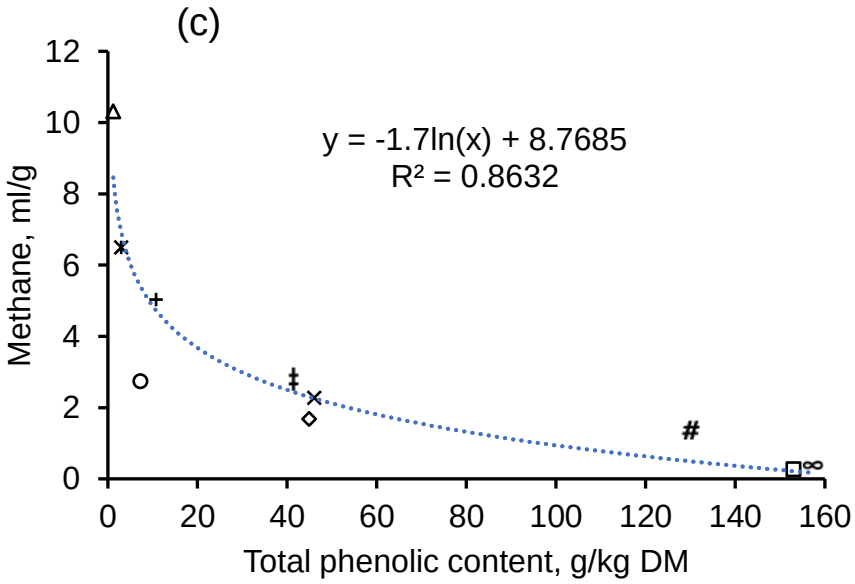

Figure 1. Effect of total phenolic content on methane production for *Acia sutherlandii* (○), *Bauhinia hookeri* (∞), *Desmanthus bicornutus* cv. JCU4 (x), *Desmanthus pernambucanus* JCU9 (‡), *Desmanthus virgatus* JCU5 ( ), *Gliricidia sepium* (+), *Neptunia monosperma* (#), *Peltophorum pterocarpum* (□), Lucerne hay ( ) and soybean meal (△) *in vitro* fermented using rumen fluid for 24 h (Tunkala et al., unpublished results).

We have focussed some of our recent research on the role of plant-derived polyphenols in reducing enteric CH_4 emissions. As a proof of concept, we have fed a polyphenol-rich sugarcane extract to sheep and observed a dose-dependent increase in daily gain and an up to 50% reduction in enteric CH_4 emissions (Prathap, 2023). While this level of GHG reduction is impressive, it still requires dietary supplementation to deliver the polyphenols and is impractical in extensive grazing systems. Therefore, we are very much interested in using tropical legumes as a means of delivering polyphenols in extensive grazing systems. *Desmanthus* is one such tropical legume that is very high in polyphenol and antioxidant activity with only moderate concentrations of tannins (Kang et al., 2022; Tunkala et al., 2023) with the potential to reduce CH_4 emissions (Black et al., 2021). In a recent *in vitro* fermentation study, we have shown that CH_4 production was negatively related to the total polyphenol content of 10 legumes (Tunkala et al., unpublished results; Figure 1). Interestingly, there was a similar relationship between in vitro rumen protein digestion and polyphenol content, suggesting that some of the mechanisms may be related to reducing rumen protein

fermentation through the formation of protein:polyphenol complexes. The *Desmanthus* species exhibited an intermediate ability to reduce CH_4 production (Figure 1) and protein fermentation, suggesting utility in reducing CH_4 while still maintaining performance. Current studies are focused on measuring enteric CH_4 emissions *in situ* in a tropical beef grazing system.

Heat Stress

HS, induced by increased environmental temperature as a result of GHG emissions, greatly impacts the productivity, health, and welfare of animals raised in both extensive and intensive production systems. Factors including feed intake, digestibility, the time digesta remains in the digestive system, and productivity are contributing factors affecting the yield and intensity of enteric CH_4 emission in ruminants (Niu et al., 2018; NASEM, 2021). These contributing factors are primarily affected when ruminant animals experience HS. Exposure to HS causes animals to consume less feed, which, combined with physiological alterations and increased energy expenditure for thermoregulation, results in impaired health and productivity (Baumgard and Rhoads, 2013). Productivity losses from HS include a slower growth rate, and reduced milk production and reproduction, leading to significant financial losses for beef and dairy producers (Thornton et al., 2022). Impairment of the immune function in heat-stressed animals increases their vulnerability to infectious diseases (Gupta et al., 2023), which is associated with the decreased productivity of the production system. This, in turn, increases CH_4 emission intensity, which is negatively related to productivity (Herrero et al., 2016). For example, the risk of mastitis infection increases in lactating cattle under HS challenge (Archer et al., 2013), which is associated with reduced milk yield, and thus increased non-CO_2 GHG emissions/L of milk produced.

From a rumen ecosystem perspective, exposure to HS is one of the causative factors for decreased rumination activity, and salivary secretion, contributing to luminal hyperosmolarity originating from the build-up of fermentation products in the rumen (Burhans et al., 2022). As a result, the absorptive function of the ruminal epithelium is impaired, increasing ruminal acidity (Aschenbach et al., 2019). The increased transport of lipopolysaccharides from the lumen into circulation through increased paracellular permeability triggers inflammation. Immune system activation is associated with increased energetic costs that negatively impact animal health, production, reproduction efficiency, and longevity, thereby contributing to elevated CH_4 emission intensity (Llonch et al., 2017).

HS can cause oxidative stress in transition dairy cows (Bernabucci et al., 2002) and other ruminants. Cyclic bouts of HS can exhaust the reduced glutathione concentration of blood and increase the oxidized glutathione concentration, resulting in oxidative stress (Lakritz et al., 2002). Hence, there is interest in using supplemental antioxidants to reduce HS susceptibility. The partitioning of blood flow away from the gastrointestinal tract and visceral organs to the periphery to increase the radiant heat loss that occurs during HS deprives the internal organs of blood flow, causing ischemia/hypoxia leading to increased production of free radicals or reactive oxygen

species (ROS) and decreased antioxidant capacity (Bernabucci et al., 2002). Normally, the free radicals produced are scavenged by cellular antioxidant systems and a balance is maintained (Chauhan et al., 2014). However, when the production of free radicals is greater than their removal by the antioxidant system, this leads to damage of macromolecules, disruption of normal metabolism and physiology and may ultimately lead to loss of cell function. Therefore, dietary antioxidant supplementation is one nutritional strategy to mitigate some of the negative impacts of HS in ruminants.

Dietary supplementation of sheep with supranutritional levels of organic selenium and vitamin E can improve the ability of sheep to handle HS (Chauhan et al., 2014; 2015; 2016; Dunshea et al., 2017). While many studies have looked at Se and other antioxidant supplementation in other ruminants, far fewer studies have been conducted under HS conditions. Encouragingly, an improvement in the preventive antioxidant systems of heat-stressed lactating dairy cows fed Se yeast has been demonstrated (Calamari et al., 2011) and it appears that organic forms of Se are more beneficial than inorganic forms (Sun et al., 2019). Also, supranutritional levels of organic selenium and vitamin E have been demonstrated to reduce the physiological effects of HS in pigs (Liu et al., 2016; Cottrell et al., 2015) and chickens (Shakeri et al., 2019). However, the level of antioxidants required is higher than allowed in many jurisdictions, so alternatives such as plant-derived polyphenols and antioxidants are being investigated. Also, as for the use of dietary supplements to reduce enteric CH_4 emissions, this approach is more difficult in extensive grazing systems. Therefore, we are currently investigating the use of grazed tropical legumes rich in polyphenols, such as *Desmanthus*, to reduce HS.

Plant Bioactives as a Mitigation Strategy

Plant bioactives could be viewed as a dietary intervention strategy to attenuate the adverse effects of HS on the health and productivity of ruminant livestock. We and others have clearly demonstrated the positive effects of plant-derived phytochemicals in chickens and pigs (Shakeri et al., 2020; Cottrell et al., 2021). In this context, Shakeri et al. (2020) found that a polyphenol-rich sugarcane extract reduced HS and improved growth performance and meat quality in broiler chickens under thermoneutral and HS conditions. New findings in a ruminant nutrition context suggest that plant bioactives may exert physiological effects on the host animals beyond their well-established effects as rumen-manipulating agents. Plant bioactives may benefit host animals through their immunomodulatory, anti-inflammatory, antimicrobial, and antioxidant effects. Some plant bioactive may also improve the integrity and functionality of the gastrointestinal tract in animals challenged with stressors (Patra et al., 2019). Some plant bioactives could stimulate appetite during HS and increase feed intake, milk production, and growth. However, some plant bioactives may negatively change the organoleptic properties of diet, which is particularly crucial in heat-stressed animals, as hyperthermia usually depresses feed intake.

An *in vitro* rumen fermentation study demonstrated the efficacy of *Capsicum* oleoresin under HS (rumen incubation temperature = 42°C) in altering the fermentation pattern towards an increased propionate-to-acetate ratio (An et al., 2022a). Several studies conducted under HS challenge have also demonstrated that adding *Capsicum* oleoresin to dairy cow diet improved feed intake (An et al., 2022b) and milk production (Abulaiti et al., 2021). The positive effects on productivity and health translate to a lower CH_4 emission intensity. A blend of *Capsicum* oleoresin and clove essential oil added to dairy cow diet at 50, 300, or 600 mg/head/d did not affect productivity but resulted in a linear reduction in enteric CH_4 yield and tended to lower CH_4 intensity as the supplementation level increased (Silvestre et al., 2022).

Concentrate Feeding as a Mitigation Strategy

One proposed strategy to combat HS is to reduce dietary fiber and increase the concentrate amount, as forage fermentation produces more heat increment (Dunshea et al., 2017; Gonzalez-Rivas et al., 2018; Prathap et al., 2021). Increasing the dietary concentrate-to-forage ratio has also been suggested as one of the effective nutritional practices to reduce enteric CH_4 emissions in ruminant livestock (Arndt et al., 2022). Feeding high-concentrate diets is easily adoptable in intensive production systems. However, both HS and high-concentrate diets are risk factors for developing subacute ruminal acidosis, which impairs animal health and productivity. These highlight the importance of measures that have the potential to attenuate the rumen function disturbances.

One issue of high-concentrate diets during HS is that if the grain component contains rapidly fermented starch as can occur with wheat or even barley, then the rapid fermentation can quickly increase rumen temperature and susceptibility to HS (Gonzalez-Rivas et al., 2016; 2017; Prathap et al., 2022). These effects of HS can be partially ameliorated by treating wheat with NaOH or by using a starch and protein binding agent to slow down the rumen fermentation of wheat starch (Gonzalez-Rivas et al., 2017; Prathap et al. 2022). Interestingly, the use of a starch and protein binding agent also decreased enteric CH_4 emissions (Prathap et al., 2023), lending credence to the use of dietary polyphenols to reduce rumen fermentation, while allowing whole tract digestion as both a HS and CH_4 mitigation strategy.

Several studies support the efficacy of plant bioactives in increasing rumination activity, modulating rumen fermentation end products, regulating rumen pH, and mitigating inflammation in dairy cows challenged with concentrate-rich diets (Kröger et al., 2017; Castillo-Lopez et al., 2021; Rivera-Chacon et al., 2022). Ricci et al. (2021) confirmed the positive effect of some phytonutrients (i.e., thymol) in the individual form in increasing salivation rate in cattle fed a high-concentrate diet, likely because of their effect on the olfactory-salivary reflex. In an *in vitro* rumen fermentation system, Khiaosa-Ard et al. (2020) evaluated the efficacy of a plant-derived alkaloid supplement [low dose: 0.088% of feed DM and high dose: 0.175% of feed DM] under different stress conditions: incubation temperature (39.5 and 42°C) and pH (6.0 and 6.6). Their findings demonstrated that the high dose decreased enteric CH_4 under the low pH challenge, but

the low dose shifted the fermentation toward increased propionate-to-acetate. Besides plant bioactives, albeit not within the scope of this review, numerous studies have demonstrated the pH-stabilizing effect of yeast products as a management tool to promote a more stable rumen fermentation in ruminants challenged with HS (Perdomo et al., 2020) or high-grain diet (Kröger et al., 2017).

Interplay of Temperature and Forage Quality Driving CH_4 Rise

Increased nutrient use efficiency has been suggested as a promising strategy to reduce CH_4 emission intensity (Llonch et al., 2017). Forages with higher nutritive value, characterized by less fiber-to-fermentable carbohydrate proportion pass through the digestive system more quickly (shorter ruminal retention time) (Knapp et al., 2014). Increased intake of more digestible forage would drive productivity and thus lower CH_4 emission intensity from enteric fermentation (Hristov et al., 2022). Feeding diets containing a greater concentration of structural carbohydrates increases the abundance and activity of fibrolytic microbiota in the rumen, driving acetate rather than propionate formation that favours ruminal methanogenesis (Patra, 2016). Shifting rumen fermentation patterns towards increased propionate formation acts as a sink of metabolic hydrogen utilization, creating an alternative to enteric CH_4 production (Wang et al., 2023). Lee et al. (2017) reported that as the environmental temperature rises, the nutritive quality of grasses declines (lower digestibility), contributing to a 0.9% increase in enteric CH_4 production for every 1°C increase in temperature. Climate is a key determinant of the nutritive quality of pasture in tropical regions and will have a larger impact on pasture-based production systems (Jayasinghe et al., 2022). The climate-driven declines in forage nutritive quality increasing ruminant CH_4 emissions signify a climate feedback loop, carrying important implications to achieve ambitious GHG reduction targets from the ruminant inventory.

Summary and Implications

Mitigating enteric CH_4 emission could decrease feed energy loss while lowering the anthropogenic contribution of ruminant livestock to GHG emission, which is a strategic step towards preventing global temperature increase. Heat stress has emerged as another major concern compromising the sustainability and profitability of livestock production systems. The stacked temperature-driven impacts on livestock productivity and CH_4 emissions poses a significant challenge to achieving GHG mitigation targets, considering the projected rise in frequency and severity of heat waves. Adopting strategies to attenuate the detrimental impacts of heat stress on the productivity and health of ruminant animals is crucial to mitigating CH_4 emissions intensity. Plant bioactives, including polyphenols, are suggested as a nutritional strategy to alleviate productivity loss, which is associated with economic returns while promoting the environmental sustainability of the production system. Increasing the dietary concentrate-to-forage ratio is an effective nutritional strategy for HS abatement while mitigating enteric CH_4 emissions although care must be taken when incorporating highly fermentable grains. This strategy may increase the risk of ruminal pH reductions, and plant bioactives may have the potential to counteract rumen function perturbations.

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The Epidemiology and treatment of Subclinical Hypocalcemia

C. R. Seely and J. A. A. McArt
Department of Population Medicine and Diagnostic Sciences
Cornell University

Introduction

The days following calving are a tumultuous time for a dairy cow as she adjusts to the metabolic and energetic demands that come along with colostrum and milk production. Of note is the appreciable increase in demand for Ca as the cow assumes lactation. Despite biological mechanisms in place to maintain Ca homeostasis, hypocalcemia befalls some cows in early lactation. Most dramatically this manifests as clinical hypocalcemia, however as our management of peripartal cows has improved, the incidence of clinical hypocalcemia has been reduced. Instead, nearly 50% of multiparous cows experience subclinical hypocalcemia; reductions in blood Ca below certain thresholds but display no physical signs of disease (Reinhardt et al., 2011). Early studies exploring subclinical hypocalcemia failed to reach a consensus as to what concentration of blood Ca was indicative of subclinical hypocalcemia and what day in milk it was best diagnosed. As such, the associations of subclinical hypocalcemia on health and production outcomes vary widely. Recent studies though have found that when the temporal patterns of blood Ca in early lactation are considered in the diagnosis of subclinical hypocalcemia, the outcomes associated with the disorder are more consistent. Parallel to the expansion in our understanding of subclinical hypocalcemia, has been the adoption of subclinical hypocalcemia mitigation and treatment strategies. While oral Ca supplementation, generally in the form of a bolus, is widely used in commercial settings, the impact that such boluses have on health, production, and reproductive parameters varies. This review aims to discuss both our current understanding of blood Ca dynamics in early lactation, how that impacts the diagnosis of subclinical hypocalcemia, and the available treatment strategies to minimize the associated negative outcomes of subclinical hypocalcemia.

Epidemiology of Subclinical Hypocalcemia

Hypocalcemia has long plagued the dairy industry as maintenance of the Ca pool during the first several days of lactation presents an appreciable challenge to the cow. As the cow assumes lactation, her demand for Ca more than doubles to support colostrum and milk production alone, and is bolstered by the essential role that Ca plays in many physiological and immunological pathways (Webb, 2003; Kimura et al., 2006; Goff et al., 2014). To meet the increased demand for Ca, several hormones and tissues work in tandem to restore and maintain normocalcemia in the hours and days following calving (Goff, 2000).

Unfortunately, some cows fail to regain normocalcemia and succumb to hypocalcemia, the reduction of blood Ca below physiologically normal concentrations. Most dramatically, this manifests as clinical hypocalcemia, arguably the most recognizable clinical diagnosis of dairy cows, as cows become recumbent soon after calving, often have cold ears, and lose the ability to raise their head or stand (McArt and Oetzel, 2023). Cases of clinical hypocalcemia, commonly referred to as milk fever, require immediate treatment with intravenous Ca to avoid death. Fortunately, over the last several decades, due to advancements in nutritional and management strategies of peripartal cows, the incidence of clinical hypocalcemia has substantially declined, affecting approximately 5% of multiparous dairy cows (Goff, 2008). However, up to 45% of multiparous dairy cows experience subclinical hypocalcemia (**SCH**) in the days following calving (Reinhardt et al., 2011).

Subclinical hypocalcemia is the occurrence of blood Ca concentrations below a given threshold at a specific time and is unaccompanied by physical signs of disease (McArt and Oetzel, 2023). Initial studies that aimed to identify SCH did so by characterizing blood Ca concentrations during the first several days of lactation and as serum Ca often reaches a nadir within the first 24 h of lactation, that was deemed the ideal time to diagnose SCH (Oetzel, 1988; Martinez et al., 2012). To improve the characterization of SCH and identify a more accurate diagnostic cutpoint of blood Ca, subsequent large field trials were conducted in which epidemiological methods were employed to explore the association of given Ca cutpoints with important outcomes such as milk production, disease events, and reproductive outcomes (Chapinal et al., 2011; Neves et al., 2018a; Venjakob et al., 2018).

With these field trials, the black box of SCH was opened; no consensus was made as to what concentration of serum total Ca (**tCa**), or day in milk to diagnose, was most accurately associated with negative health and production outcomes. Diagnostic cutpoints of tCa ranged from 1.8 to 2.2 mmol/L and the day in which blood was analyzed varied from 0 to 7 days in milk (Couto Serrenho et al., 2021). When Chamberlin et al. (2013) classified multiparous Holstein cows as SCH when blood ionized Ca was <1.0 mmol/L (approximately 2.0 mmol/L tCa) within the first 24 h of calving, they saw no difference in the incidence of clinical mastitis, ketosis, metritis, displaced abomasum, or reproductive outcomes between SCH cows and those that remained normocalcemic. Additionally, there was no difference in milk production for the first 35 DIM between SCH and normocalcemic cows. When Neves et al. (2018) explored the association of tCa concentrations of multiparous Holstein cows within 12 h of calving, they found that cows with tCa ≤1.85 mmol/L were at increased risk for developing a displaced abomasum compared to those with tCa above the cutpoint. Interestingly, they also found that multiparous cows with tCa ≤1.95 mmol/L produced more milk than cows with tCa above the given cutpoint. Furthermore, in a large field trial conducted on 55 herds across North America in which SCH was diagnosed as tCa <2.1 mmol/L at any point during the first week of lactation, they found that SCH cows were at increased odds of developing a displaced abomasum and produced less milk than normocalcemic cows.

In studies where the temporal patterns of blood Ca during the first serval days of lactation were considered in the diagnosis of SCH, a clearer picture of SCH emerged, and the associated negative impacts of SCH were more consistent. When only 1 DIM tCa concentrations were considered, Neves et al. (2018b) observed no association between SCH diagnosis and risk of metritis, however they found that if parity 2 cows had tCa ≤1.97 mmol/L at 2 DIM and parity 3 cows had tCa ≤2.2 mmol/L at 4 DIM, they were at increased risk for developing metritis, displaced abomasum, or both compared to cows that remained above the stated cutpoints. Furthermore, they observed that reduced blood tCa at 1 DIM was associated with greater milk production while reduced blood tCa at 4 DIM was associated with decreased milk production. Caixeta et al. (2017) also found that cows with tCa ≤2.15 mmol/L for the first 3 DIM took significantly longer to return to cyclicity and had decreased odds of becoming pregnant to first service compared to cows that only had 1 sample ≤2.15 mmol/L or that remained above the cutpoint during the first 3 DIM.

These studies indicated that the absolute nadir of tCa in early lactation may not be indicative of associated negative outcomes, but rather the timing and persistency of SCH should be considered. McArt and Neves (2020) further investigated the dynamics of tCa during the first 4 DIM by classifying multiparous Holstein cows (n = 263) into 1 of 4 SCH groups based on tCa at 1 and 4 DIM. The tCa cutpoints were identified through receiver operator characteristic curves established by Neves et al. (2018b) and were ≤1.77 mmol/L and ≤2.20 mmol/L for 1 and 4 DIM, respectively. If cows had tCa above both cutpoints at 1 and 4 DIM, they were deemed normocalcemic (**NC**; n = 109); cows with tCa ≤1.77 mmol/L at 1 DIM but >2.20 mmol/L at 4 DIM were classified as transient SCH (**tSCH**; n = 50), those that were below the cutpoints at both 1 and 4 DIM were classified as persistent SCH (**pSCH**; n = 34), and finally those with tCa >1.77 mmol/L at 1 DIM but ≤2.20 mmol/L at 4 DIM were deem delayed SCH (**dSCH**; n = 70). The dynamics of tCa during the early lactation period are outlined in Figure 1. When the risk of adverse events, defined as hyperketonemia, metritis, displaced abomasum, herd removal, or a combination thereof during the first 60 DIM, was explored in these cows, the authors found that pSCH and dSCH cows were nearly twice as likely to experience an adverse event compared to NC cows. Furthermore, tSCH cows produced significantly more milk throughout the first 10 wk of lactation compared to NC cows.

To further explore the etiology of SCH and the dynamics of tCa during early lactation, Seely et al. (2021) again classified multiparous Holstein cows (n = 78) as NC, tSCH, pSCH, or dSCH based on tCa at 1 and 4 DIM (1 DIM cutpoint: tCa ≤1.95 mmol/L and 4 DIM cutpoint: tCa ≤2.20 mmol/L) and measured daily dry matter intake (**DMI**) from 14 days prepartum to 21 days postpartum. During the prepartum period, DMI was similar between SCH groups ($P = 0.6$), however following parturition DMI was significantly different between SCH groups ($P < 0.001$) with NC and tSCH cows consuming more feed than their pSCH and dSCH counterparts (NC = 20.9 ± 1.0 kg/d, tSCH = 21.2 ± 1.0 kg/d, pSCH = 17.5 ± 1.2 kg/d, and dSCH = 18.6 ± 1.0 kg/d). Of note, was the unique pattern of DMI during the first 4 DIM between SCH groups that closely mirrored the temporal pattern of blood tCa occurring simultaneously, further highlighting the unique nature of tCa concentrations during the early lactation period.

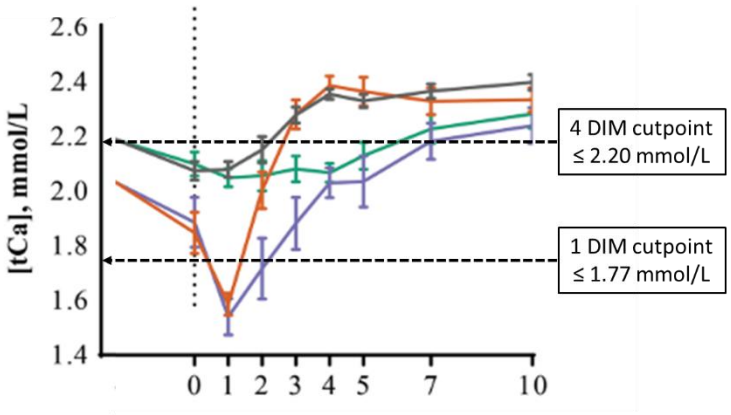

Figure 1. Dynamics of tCa for multiparous Holstien cows (n = 263). Cows were classified as follows: normocalcemic (NC, gray line; [Ca] >1.77 at 1 DIM and 2.20 mmol/L at 4 DIM); transient subclinical hypocalcemia (tSCH, orange line; [Ca] ≤1.77 at 1 DIM and >2.20 mmol/L at 4 DIM); persistent subclinical hypocalcemia (pSCH, purple line; [Ca] ≤1.77 at 1 DIM and ≤2.20 mmol/L at 4 DIM); or delayed subclinical hypocalcemia (dSCH, green line [Ca] >1.77 at 1 DIM and ≤2.20 mmol/L at 4 DIM). Adapted from McArt and Neves (2020).

The culmination of findings of McArt and Neves (2020) and Seely et al. (2021) as well as earlier work by Caixeta et al. (2017) and Neves et al. (2018b) considering the dynamic patterns of tCa during early lactation in the diagnosis of SCH demonstrate homogeneous results. Together, these works suggest that a transient drop in blood Ca at 1 DIM may be necessary as the cow prepares for the demands of lactation, while low blood Ca at 3 and 4 DIM is representative of a larger metabolic disruption, putting the cow at increased risk for additional disease development and decreased reproductive success and production. Considering the consistent negative production and health outcomes associated with low tCa at 4 DIM, we postulate that these cows are experiencing a state of dyscalcemia where the demands of early lactation have outpaced biological mechanisms in place to restore and maintain tCa concentrations.

To explore the associated impact of dyscalcemia on reproductive outcomes, Seely and McArt (2023) classified multiparous Holstein cows (n = 697) as dyscalcemic if 4 DIM tCa ≤2.20 mmol/L (n = 182) or normocalcemic if 4 DIM tCa >2.20 mmol/L (n = 515). They found that the odds of becoming pregnant to first service was significantly reduced in dyscalcemic cows compared to normocalcemic cows (odds ratio = 0.75; P = 0.01). While 27.4% of normocalcemic cows became pregnant to first service, 18.1% of dyscalcemic cows became pregnant to first service. Similarly, the median time to pregnancy was longer for dyscalcemic cows (119 ± 16 d) compared to normocalcemic cows (103 ± 11d; P = 0.1). The hazard of pregnancy by 150 DIM was also reduced in dyscalcemic cows (incidence = 65.4%; hazard ratio = 0.82; P = 0.06) compared to normocalcemic cows (incidence = 70.7%).

Beyond the established negative associations that dyscalcemia has on health and production, low blood Ca concentrations at large can negatively impact rumen function and rumination time. The strength and speed of rumen contractions rely on cytosolic Ca (Webb, 2003). In cases of clinical hypocalcemia, it is not uncommon for rumen contractions to cease (Jørgensen et al., 1998), and (Goff et al., 2020) reported a positive association between rumination time and blood Ca during the first 2 DIM. In an effort to explore the association between dyscalcemia and rumination time during the periparturient period and perhaps identify an alternative to blood sampling for the diagnosis of dyscalcemia, Seely and McArt (2023) recorded rumination and activity time for multiparous Holstein cows (n = 182). Cows were classified as dyscalcemic if 4 DIM tCa ≤2.20 mmol/L (n= 57) or normocalcemic if 4 DIM tCa >2.20 mmol/L (n = 125) and rumination and activity time was recorded for the 14 d before and after calving. While there was no difference in rumination or activity time prepartum between dyscalcemic and eucalcemic cows (both $P > 0.3$), postpartum rumination and activity times were both significantly reduced in dyscalcemic cows compared to normocalcemic cows (both $P < 0.01$). Dyscalcemic cows ruminated an average of 480.5 ± 15 min/d while normocalcemic cows ruminated an average of 512.3 ± 10.5 min/d during the first 14 DIM. Dyscalcemic cows were also less active than normocalcemic cows (407.8 ± 15.5 arbitrary units/d versus 436.1 ± 11.0 arbitrary units/d). Predictive models utilizing rumination and activity variables recorded during the first 4 DIM also showed promise in correctly identifying dyscalcemic cows (best performing model: sensitivity = 38.6%, specificity = 94.4%, accuracy = 77.0%).

Recent works exploring the associations of dyscalcemia challenge the traditional framework of SCH diagnosis and Figure 2 summarizes the outcomes associated with the temporal patterns of blood Ca during early lactation. What remains to be elucidated though is the driving force that allows some cows to regain normocalcemia after a transient drop in blood Ca at 1 DIM, while reductions in blood Ca persist or do not drop until 4 DIM in others. Furthermore, despite our improved understanding of blood Ca dynamics following parturition, hypocalcemia still befalls many dairy cows in early lactation and for that we turn to a variety of treatment and prevention strategies.

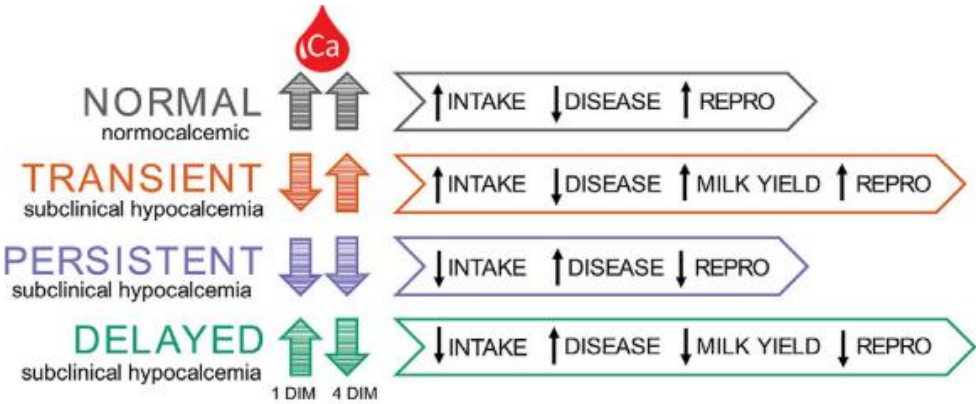

Figure 2. Calcium dynamic groups based on blood total calcium concentrations of multiparous cows at 1 and 4 DIM and their association with dry matter intake, subsequent disease incidence, early lactation milk yield, and reproductive success.

Treatment of Subclinical Hypocalcemia

Management strategies aimed at supporting blood Ca concentrations during the early lactation period are commonplace in high producing dairy herds in North America. Supplemental Ca can be provided in several forms; intravenous or subcutaneous infusion of readily available Ca, or oral administration of Ca salts in the form of liquid, paste, or bolus. Intravenous infusion of Ca elicits a rapid and robust increase in blood Ca, however within hours of infusion, blood Ca falls and remains low for the hours and days to follow (Braun, 2009; Blanc et al., 2014). While intravenous Ca infusion is necessary for the treatment of clinical milk fever, it is not appropriate for cows with SCH as the dramatic increase and subsequent decrease in blood Ca may further inhibit the return to normocalcemia by impeding Ca homeostatic mechanisms. Oral Ca supplementation may be more appropriate for the treatment and prevention of SCH as it yields a moderate, but sustained increase in blood Ca (Domino et al., 2017).

Due to the relative ease of administration, oral Ca boluses have become a common prophylactic strategy to mitigate SCH. Several Ca boluses are commercially available and are generally administered in 2 doses, the first at calving and the second 12 to 24 h later. Despite their widespread use in commercial settings, there are a limited number of scientific reports that explore the effects of oral Ca boluses on health and production outcomes, and those that have report varied results. When explored as a prophylactic treatment, administered at the herd level to all early lactation cows, oral Ca boluses have minimal effects on milk production, health outcomes, and reproductive measures (Oetzel and Miller, 2012; Valldecabres et al., 2023). The neutral effect that Ca boluses have on health and production outcomes at the population level indicates that while some cows may benefit from supplementation, others realize no gains or may even be detrimental to their success.

At the population level, there is little evidence for a difference in milk production or reproductive measures between cows given oral Ca boluses at and around calving and those that are not supplemented (Domino et al., 2017; Valldecabres et al., 2023). However, subpopulations of cows appear to respond differently to oral Ca supplementation as Oetzel and Miller (2012) reported significantly increased milk production in cows with above-average production in the previous lactation that were supplemented with an oral Ca bolus at 0 and 24 h of lactation compared to their high producing herdmates who were not supplemented. In the same study, cows with below average production produced less milk when given an oral Ca bolus after calving compared to non-supplemented, low producing cows. Other reports also suggest that lame cows or those with high (≥3.5) body condition score at calving are at reduced risk for experiencing a negative health event in early lactation when they are supplemented with oral Ca at or around calving (Oetzel and Miller, 2012; Leno et al., 2018). Interestingly though, Leno et al. (2018) observed an increased risk for risk for negative health events in parity 2 cows that were given an oral Ca bolus at calving compared to parity 2 cows that were not supplemented. Similarly, Martinez et al. (2016a;b) reported reduced reproductive success and increased risk of disease in primiparous cows given oral Ca boluses in early lactation.

Oral Ca boluses elicit a slow and sustained increase in blood Ca over the course of 8 to 24 h following administration and there is little impact of supplementation on blood Ca concentration 48 h post administration (Martinez et al., 2016a; Domino et al., 2017; Frost et al., 2022). The differential effects that oral Ca bolus supplementation at 0 and 1 DIM has on health and production outcomes may be due to the varying nature of Ca dynamics in the days following parturition. The traditional bolus supplementation strategy was implored to target the nadir in blood Ca that often occurs within 24 h of parturition, however as our understanding of early lactation blood Ca dynamics has grown and the phenomenon of dyscalcemia has emerged, more targeted strategies to support blood Ca and minimize the associated negative impacts of dyscalcemia are required.

Seely et al. (2022) sought to explore the impact of oral Ca bolus timing on milk production and health events and blood Ca dynamics in the early lactation period in an effort to target cows with dyscalcemia. They enrolled multiparous Holstein cows (n = 998) at the time of calving and cows were randomly assigned to 1 of 3 treatments: 1) control; no supplemental Ca at or around the time of parturition (**CON**; n = 343), 2) conventional bolus; conventional administration of an oral Ca bolus at the time of calving and again 24 h later (**BOL-C**; n = 330), or 3) delayed bolus; delayed administration of an oral Ca bolus at 48 and 72 h post-calving (**BOL-D**; n = 325).

As with previous reports exploring the effects of oral Ca boluses, Seely et al. (2022) reported differential results. At the study population level, there was no evidence for a difference in milk production for the first 10 wk of lactation between treatments (P = 0.2), however a parity × treatment effect was evident (P = 0.002). There was no evidence for a difference in milk production between treatment groups for parity 2 cows, however parity 3 cows that received oral Ca at 2 and 3 DIM produced significantly more milk than BOL-C treated cows and CON cows (BOL-D: 52.0 ± 1.3 kg/d, BOL-C: 47.9 ± 1.6 kg/d, CON: 49.8 ± 1.5 kg/d; P = 0.003). Interestingly, this improvement in milk production by BOL-D treatment did not appear in parity ≥4 cows and milk production was similar between treatments. Furthermore, the incidence of adverse events (metritis, displaced abomasum, herd removal, or a combination thereof within the first 30 DIM) was similar between treatment groups (BOL-D: 11.5%, BOL-C: 8.0%, CON: 10.8%; P = 0.4). Similarly, while controlling for parity group, there was no evidence for a difference in serum tCa or incidence of dyscalcemia (defined as 4 DIM serum tCa ≤2.20 mmol/L) between treatment groups (BOL-D: dyscalcemia incidence 32%, [tCa] 2.09 ± 0.02 mmol/L, BOL-C: dyscalcemia incidence 28%, [tCa] 2.11 ± 0.02 mmol/L, CON: dyscalcemia incidence 26%, [tCa] 2.11 ± 0.02 mmol/L).

The results of the Seely et al. (2022) study are not unlike previous reports exploring the impact of oral Ca bolus supplementation on health and production outcomes, further supporting the idea that postpartum oral Ca supplementation differentially impacts cohorts of cows. In culmination with findings reported by Martinez et al. (2016a) and Leno et al. (2018) it is likely that parity ≤2 cows do not require Ca supplementation after calving as they are able to efficiently navigate the Ca challenge of

early lactation through homeostatic pathways as milk production is not as great as advanced parity cows. Alternatively, despite minimal impact of blood Ca, delaying oral Ca supplementation to 2 and 3 DIM in parity 3 cows appeared to positively impact milk production and may offer a promising strategy to mitigate dyscalcemia. While parity ≥4 cows saw no benefit from delayed or conventional bolus supplementation, older cows are at increased risk for SCH and more appropriate Ca supplementation strategies are needed for older cows. Perhaps advanced parity cows require larger doses of supplementation Ca or for a longer duration? Regardless, future work should be dedicated to optimizing the prevention and treatment of SCH and dyscalcemia in advanced parity cows with focus given to Ca dynamics in early lactation.

Conclusions

Hypocalcemia has been an ever-present threat to dairy cows. Over the years we have reduced the incidence of clinical milk fever and instead have focused on the subclinical manifestation of the disorder. With that, other challenges have arisen such as when to diagnose and what threshold of blood Ca is associated with negative health and production outcomes. With an improved understanding of the dynamics of blood Ca in early lactation, we have come to realize that the timing and persistency of reduced blood Ca may be more indicative of associated negative outcomes than the absolute nadir of blood Ca. Through a series of large field trials, it has become apparent that reductions in blood Ca at 4 DIM are consistently associated with decreased milk production, decreased intake, increased risk for negative health events, and reduced reproductive success and has since been coined dyscalcemia. Regardless of how and when subclinical hypocalcemia or dyscalcemia is diagnosed, treatment and prevention strategies aimed at supporting blood Ca in early lactation are widely employed across the dairy industry. Traditionally administered at 0 and 1 DIM, oral Ca boluses are commonplace in the commercial setting however have varied impacts on production and health outcomes. Recent works have explored the effects of delaying oral Ca bolus supplementation to 2 and 3 DIM, to reduce the associated impacts of dyscalcemia and resulted in improved milk production in parity 3 cows. Future work is needed to better understand the etiology of dyscalcemia and what allows certain cows to regain normocalcemia after a transient drop in blood Ca at 1 DIM. Knowing this may inform us of more optimized treatment and prevention strategies that will minimize the negative impact that dyscalcemia has on health and production and improve the productive potential of the dairy industry.

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Vitamin D and Hypocalcemia in the Dairy Cow

C. D. Nelson
Department of Animal Sciences
University of Florida

Introduction

Vitamin D is a common nutrient in the diets of dairy cows that does not receive much attention because it is inexpensive, and it uses little space in the ration. Cows are efficient at cutaneous vitamin D_3 synthesis when outdoors during summer months (Hymoller and Jensen, 2010) and they acquire some vitamin D_2 from forages (Horst and Littledike, 1982) but for practical purposes (i.e., housing and seasonal variation) nearly all dairy cows in the U.S. receive supplemental vitamin D_3. The hormonal activity of vitamin D is required for normal Ca homeostasis and, accordingly, there have been many attempts to prevent or treat hypocalcemia using vitamin D metabolites. Dietary vitamin D_3 recommendations were recently updated for dairy cattle (NASEM, 2021) but the revisions have few practical implications for formulating diets. The 25-hydroxyvitamin D_3 [25(OH)D_3, calcidiol] metabolite, commercially marketed as Hy-D®, has recently been approved for use ruminant diets. Feeding 25(OH)D_3 during late gestation increases total tract Ca absorption and has positive effects for transition cow performance (Martinez et al., 2018, Poindexter et al., 2023a). Herein, vitamin D physiology and its role in prevention of hypocalcemia will be discussed along with updates for vitamin D nutrition and nutritional interventions that benefit vitamin D metabolism of transition dairy cows.

Vitamin D Physiology

Vitamin D is the collective term for a class of seco-steroid molecules originally discovered to have anti-rachitic activity. Vitamin D_3 and its metabolites are derived from 7-dehydrocholesterol in animals through a process of photoconversion in the skin (Holick et al., 1981). The process is efficient in cattle and the vitamin D_3 metabolites represent 90 to 95 % of vitamin D in cattle. Vitamin D_2 is derived from ergosterol of fungi and represents a small but appreciable fraction of vitamin D metabolites in cattle. Vitamin D_2 and vitamin D_3 must undergo subsequent enzymatic oxidation steps to become activated and exert most activity through intracellular vitamin D receptors. Although the rates and transport of vitamin D_2 and vitamin D_3 metabolism differ somewhat in cattle (Sommerfeldt et al., 1983, Hymøller and Jensen, 2017), they share the same enzymes and receptors for activity and the focus will be on vitamin D_3 from here on.

An overview of the vitamin D pathway and some outcomes of endocrine and intracrine vitamin D signaling are depicted in Figure 1. Oxidation of vitamin D_3 to 25(OH)D_3 is catalyzed by several hepatic 25-hydroxylases. Subsequent oxidation of 25(OH)D_3 to 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$$D_3$, calcitriol], the active metabolite, is catalyzed by the 25-hydroxyvitamin D_3 1α-hydroxylase (Fraser and Kodicek, 1973). Finally, oxidation of 25(OH)D_3 and 1,25(OH)$_2$$D_3$ metabolites to 24,25-dihydroxyvitamin D_3

and 1,24,25-trihydroxyvitamin D_3, respectively, is catalyzed by the 25-hydroxyvitamin D-24-hydroxylase. The 24-hydroxyvitamin D metabolites are reported to have some biological activity, but are generally regarded as degradation products of vitamin D (Sakaki et al., 2005).

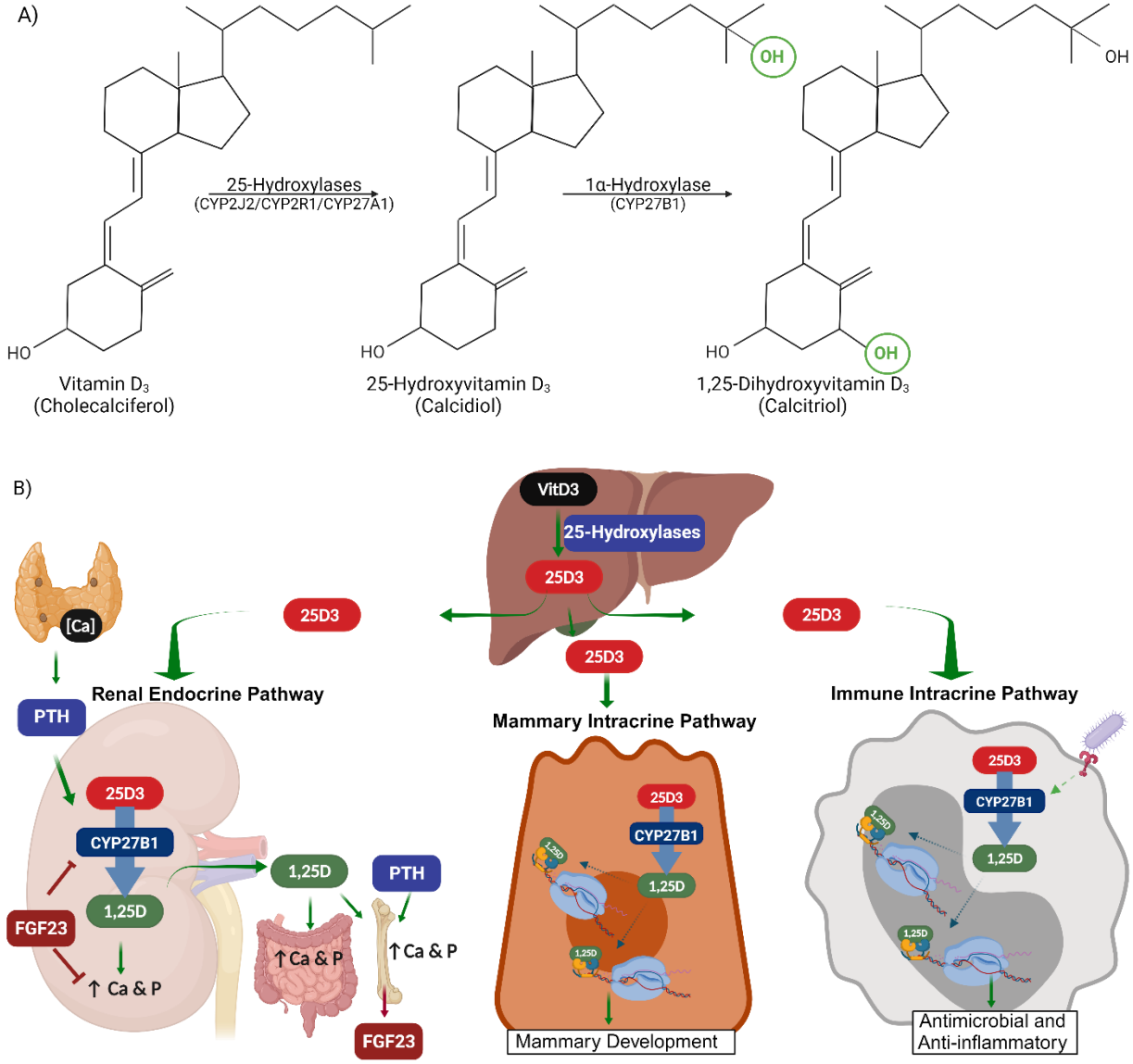

Figure 1. Oxidation of vitamin D_3 to 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 (A) and examples of vitamin D pathways (B).

The main points of control in the vitamin D metabolic pathway center around regulating the concentration of $1,25(OH)_2D_3$. The $1,25(OH)_2D_3$ concentration of plasma is typically 20 to 50 pg/mL in lactating and dry cows, and upwards of 100 to 300 pg/mL in 2 to 3 DIM. Plasma $1,25(OH)_2D_3$ does not correspond to vitamin D intake (Poindexter et al., 2023b). Most 1α- hydroxylase activity occurs in the kidneys under strict hormonal control,

but a small fraction also occurs in adipocytes, immune cells, mammary epithelial cells, and reproductive tissues under control of various signal processes. The $1,25(OH)_2D_3$ induces its own catabolism by upregulation of 24-hydroxylase in a classical feed-back manner. The 24-hydroxylase can be expressed in nearly every cell that has vitamin D receptors, which serves to control vitamin D activity at the cellular level. Collectively, the balance of 1α-hydroxylase and 24-hydroxylase activity serve to regulate vitamin D activity.

In comparison to $1,25(OH)_2D_3$, the $25(OH)D_3$ metabolite is the major form of vitamin D circulating in blood and, with a half-life of approximately two weeks, its concentration in plasma serves as the best indicator of vitamin D status. Plasma $25(OH)D_3$ concentrations from 40 to 100 ng/mL are typically observed in dairy cows fed standard rations (Nelson et al., 2016a). Similar concentrations are observed for beef cows on summer pasture (Nelson et al., 2016b). Some control of the $25(OH)D_3$ concentration also exists, albeit to a lesser extent than control of $1,25(OH)_2D_3$. Cutaneous vitamin D_3 synthesis is limited by feedback signals effectively limiting the concentration of $25(OH)D_3$ in circulation (Holick et al., 1981). In cattle, the upper limit of serum $25(OH)D_3$ derived from sun exposure seems to be near 100 ng/mL based on observed serum $25(OH)D_3$ concentrations in beef cows (Nelson et al., 2016b). Some feedback inhibition also seems to exist in oxidation of vitamin D_3 to $25(OH)D_3$ in dairy cows (Rodney et al., 2018, Poindexter et al., 2020, Poindexter et al., 2023b). For example, increasing the rate of dietary vitamin D_3 from 1 mg/d (40,000 IU/d) to 3 mg/d (120,000 IU/d) did not increase plasma $25(OH)D_3$ concentrations even though it increased plasma vitamin D_3 concentrations (Figure 2). Consequently, there is little benefit, and potentially detrimental consequences, to overfeeding vitamin D_3 (Fraser, 2021).

Nearly all vitamin D metabolites circulate in blood bound to the vitamin D binding protein (DBP), an abundant member of the albumin family of proteins (Haddad et al., 1993). Significant genetic variation exists for the group-specific component (*GC*) globulin gene encoding the vitamin D binding protein and it, along with genetic variants of genes encoding 25-hydroxylases, is another key determinant in circulating concentrations of $25(OH)D_3$ (Delanghe et al., 2015). In regard to hypocalcemia of dairy cows, polymorphisms linked to the *GC* gene in cattle are associated with risk of postpartum hypocalcemia (Cavani et al., 2022). The basis for the association between *GC* variants and hypocalcemia is not yet understood, but knowledge of the association allows for selection of animals with lower risk of hypocalcemia.

Biological activity of vitamin D is exerted primarily through intracellular vitamin D receptors (VDR). The VDR is a member of the nuclear hormone family of receptors, which have DNA-binding and ligand-binding domains and largely function as transcription factors (Pike et al., 2007). The DNA-binding domain of the VDR recognizes short, specific sequences of DNA referred to as vitamin D response elements. The elements are located promoter or enhancer segments upstream, downstream, and within vitamin D target genes. Thousands of genes are under control of vitamin D response elements, but binding of the VDR to a given element is largely limited by cell-type, differentiation stage, and co-signaling molecules (Carlberg, 2014). Some well-known gene targets of the VDR include those involved in Ca binding and transport like the genes encoding intestinal transient

receptor potential family vanilloid subgroup 6 (TRPV6) and calbindin-D9k proteins. Additionally, there are the many extra-calcemic vitamin D target genes such as those related to anti-inflammatory and anti-microbial functions in immune cells and mammary gland development (Nelson et al., 2018).

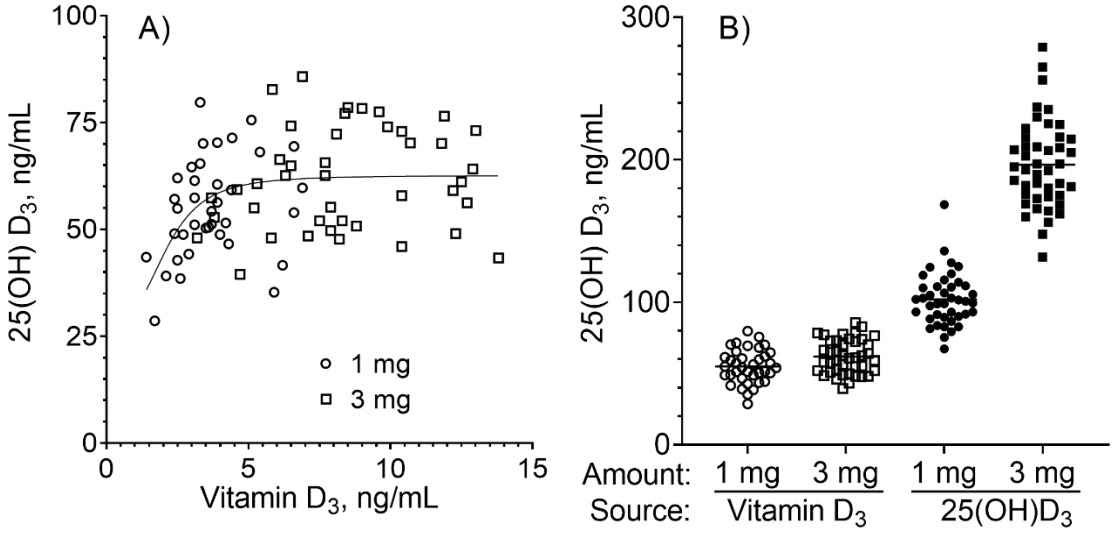

Figure 2. Holstein cows (n = 173) were fed either 1 or 3 mg/d of vitamin D_3 or 25(OH)D_3 for the last 4 weeks of gestation. A) Plot of serum 25(OH)D_3 vs. serum vitamin D_3 in cows fed 1 or 3 mg/d vitamin D_3. Effect of amount on serum vitamin D_3, $P < 0.01$; effect of amount on serum 25(OH)D_3, $P > 0.1$. B) Plot of serum 25(OH)D_3 concentrations for cows fed 1 or 3 mg/d of vitamin D_3 or 25(OH)D_3. Effect of source, $P < 0.01$; effect of amount, $P < 0.01$; Interaction between source and amount, $P < 0.01$.

Non-genomic actions of vitamin D also are reported and may have particular significance for gastrointestinal Ca absorption. Rapid influx of Ca in response to 1,25$(OH)_2D_3$ was observed in intestinal epithelial cells, and non-genomic responses were confirmed by knockdown of nuclear VDR in bone cells (Nemere et al., 2012). Membrane-associated VDR and membrane protein disulfide isomerase family A member 3 (PDIA3), which has several aliases [1,25$(OH)_2D_3$-MARRS, GRP58 and ERp57) are reported to be responsible for the non-genomic actions of 1,25$(OH)_2D_3$. The non-genomic vitamin D pathway involves activation of protein kinase A (PKA) and PKC intracellular pathways and results in rapid uptake of Ca and P. Despite the potential relevance of the non-genomic vitamin D pathway for postpartum Ca and P absorption, it has yet to be defined in cattle.

Vitamin D Endocrine Response to Hypocalcemia

The classical vitamin D endocrine system exists as part of the intricate endocrine system that controls blood and skeletal Ca and P economies (Figure 1). Case in point, 200 µg of 1,25$(OH)_2D_3$ within 6 h of parturition increased increase in plasma Ca by 0.3 m*M* and plasma P by 2 m*M* compared with control (Vieira-Neto et al., 2021b). The 1,25$(OH)_2D_3$ is one of several hormones that function in a concerted manner to maintain

blood Ca and P concentrations within a very narrow range and support skeletal development and homeostasis (Horst et al., 2005). Control of Ca is especially critical because of the various (nerve, muscle, etc.) signaling processes that involve the Ca^{2+} ion. Accordingly, small deviations in blood Ca from normal have severe, even fatal, consequences.

Calcium sensing receptors in the parathyroid glands serve as the primary thermostat for regulating the extracellular pool of Ca^{2+}. Decreased blood Ca^{2+} triggers the release of parathyroid hormone (PTH) as evident by the increased serum PTH concentration of postpartum dairy cows (Rodney et al., 2018). The PTH stimulates renal $1,25(OH)_2D_3$ synthesis and, along with $1,25(OH)_2D_3$, stimulates osteoclasts to release Ca and P from bone. The $1,25(OH)_2D_3$, meanwhile, stimulates gastrointestinal Ca and P absorption and renal reabsorption. The calcitropic and phosphotropic activities of PTH and $1,25(OH)_2D_3$ are counteracted by calcitonin and fibroblast growth factor 23 (FGF23). Calcitonin and FGF23 have not been studied much in cattle, so most of what we know is from other species. Calcitonin is secreted by C-cells of the thyroid gland in response to elevated blood Ca. Calcitonin suppresses intestinal Ca absorption acts in the kidneys to suppress Ca reabsorption. During normocalcemic conditions calcitonin promotes bone mineralization by stimulating renal $1,25(OH)_2D_3$ synthesis and inhibiting bone resorption but during hypercalcemic conditions calcitonin inhibits 24-hydroxylase activity (Beckman et al., 1994). FGF23 is released from bone cells in response to elevated P and $1,25(OH)_2D_3$ (Shimada et al., 2004). FGF23 suppresses renal phosphate transporters and $1,25(OH)_2D_3$ synthesis, thereby counteracting elevated P. In addition to the classical Ca and P regulating hormones described above, serotonin and PTH-related protein (PTHrp) from the mammary gland also have a significant contribution in Ca and skeletal homeostasis (Weaver and Hernandez, 2016). It is important to keep in mind these hormones function as a delicate system among multiple organs to maintain Ca, P, and skeletal homeostasis.

The irreversible loss of Ca to milk at the onset of lactation require a tremendous shift in Ca and P economies that rely on appropriate hormone response for the cow to adapt. Failure of any aspects of the hormone system results in hypocalcemia and hypophosphatemia. The consequences of clinical hypocalcemia resulting from maladaptation to the onset of lactation are evident. Subclinical hypocalcemia also impairs immunity, smooth muscle function, and insulin signaling in cows. Delayed or chronic subclinical hypocalcemia specifically are associated with poor lactation and reproductive performance (Neves et al., 2018, Wilkens et al., 2020). Complicating the situation even more are the perturbations caused by inflammation. Inflammation associated with diseases like metritis result in temporal sequestration of Ca by tissues, decreased feed intake and impaired gastrointestinal function (Horst et al., 2021). Careful management of transition cows and transition cow diets are necessary for prevention of maladaptation to the demand of Ca at the onset of lactation.

Nutritional Strategies to Enhance Vitamin D Metabolism

The NASEM 2021 Nutrient Requirements for Dairy Cattle updated the recommendations for supplemental vitamin D_3. The recommended amounts of supplemental vitamin D_3 were increased slightly for dairy calves, heifers and cows. A summary of the updated recommendations is provided in Table 1. It should be noted that data from dose-response experiments were not available to develop the vitamin D recommendations; instead, the recommendations represent the committee's best estimate of adequate intake. The revised recommendations have little to no impact on formulating diets because most nutritionists have been formulating diets to include more than the recommended amount of vitamin D_3 for many years (Weiss, 1998). Most lactating cow diets provide cows 30,000 to 50,000 IU (0.75 to 1.25 mg) supplemental vitamin D_3 per day. Likewise, dry cow and closeup cow diets usually provide at least 20,000 to 30,000 IU supplemental vitamin D_3 per day.

The average serum 25(OH)D concentration of 700 samples collected from cows in multiple herds under various management and geographical locations in the U.S. was 68 ng/mL with 90% of those samples between approximately 40 and 100 ng/mL regardless of season, housing or geographical location (Nelson et al., 2016a). Under these practices, it is unlikely that postpartum hypocalcemia results from inadequate vitamin D. Nonetheless, uncertainty still exists for the optimal amount of vitamin D_3, particularly for transition cows, because there is a lack of experimental data to form solid recommendations. Supplementing high (> 50,000 IU/d, or 1.25 mg/d) amounts of vitamin D_3 is not beneficial, and potentially detrimental, because there is a limit in the uptake and conversion of vitamin D_3 to 25(OH)D_3 in cows (Figure 2). Whether an optimum amount between 20,000 to 50,000 IU (0.5 to 1.25 mg) of vitamin D_3 per day remains to be determined.

Technical note for units of measure: Vitamins were historically quantified based on biological activity (anti-rachitic activity in the case of vitamin D) and international units were defined to standardize activity. Unfortunately, international units are still widely used today even though the unit of measure results in much confusion and misuse. For example, vitamin D_2 is metabolized somewhat differently in cattle and although it contributes to overall vitamin D activity 1 mg vitamin D_2 does not have the same activity as 1 mg vitamin D_3 (Sommerfeldt et al., 1983). Therefore, units for vitamin D_3 in Table 1 are provided as micrograms and international units. Either mass or molar units should be used for comparison of vitamin D_3 with other sources of vitamin D.

Supplemental 25(OH)D_3 was recently approved for ruminant diets in the U.S. Also referred to as calcidiol and commercially marketed as Hy-D®, this vitamin D metabolite has been fed to poultry for many years because it is more readily absorbed compared with vitamin D_3. In addition to more efficient absorption, direct feeding of 25(OH)D_3 bypasses the initial hepatic oxidation (Figure 1) step making it much more effective at increasing serum 25(OH)D_3 compared with feeding vitamin D_3. For example, feeding 3 mg of 25(OH)D_3 to closeup cows increased serum 25(OH)D_3 from 60 ng/mL to 200 ng/mL, whereas feeding 3 mg vitamin D_3 did not cause serum 25(OH)D_3 to increase above 100

ng/mL (Figure 2; Poindexter et al., 2023b). Feeding 3 mg of 25(OH)D_3 per day for the last 3 to 4 weeks of gestation increased total tract digestibility of Ca, colostrum yield, and milk yield compared with feeding vitamin D_3 (Martinez et al., 2018, Silva et al., 2022, Poindexter et al., 2023a). Although feeding 25(OH)D_3 did not increase serum Ca at 0 or 1 DIM compared with vitamin D_3, it did increase average serum Ca from 2 to 11 DIM (Poindexter et al., 2023b). The increased serum Ca at 2 to 11 DIM from feeding 25(OH)D_3 was correlated with serum 25(OH)D_3 and milk yield of cows (Poindexter et al., 2023a).

Table 1. Vitamin D_3 recommendations for dairy and beef cattle[1].

Stage[2]	µg/kg BW	µg/kg DM	IU/kg DM	IU/lb. DM	IU/d
Dry	0.75	40	1,600	726	22,500
Closeup	0.75	50	2,000	908	22,700
Fresh	1.0	45	1,800	817	28,000
Lactating	1.0	30	1,200	545	28,000
Calves	0.8	80	3,200	1,453	3,200
Heifers	0.75	50	2,000	908	12,000
Beef cows	0.165	6.9	275	125	5,000

[1] Based on NASEM Nutrient Requirements for Dairy Cattle (2021) and NRC Nutreint Requirements for Beef Cattle (2016).
[2] Estimated for typical lactating, dry, closeup or fresh Holstein cow, pre-weaned Holstein calf and growing Holstein heifers. 1,000 IU = 25 µg vitamin D_3.

The benefit of feeding 25(OH)D_3 for transition cow performance also may be attributed to the immunomodulatory role of vitamin D. Vitamin D signaling promotes anti-inflammatory, antimicrobial and antioxidant functions of immune cells (Nelson et al., 2018). Feeding 25(OH)D_3 compared with vitamin D_3 had wide-spread effects on expression of genes with cell-signaling, host-defense and leukocyte recruitment in immune cells of dairy cows (Vieira-Neto et al., 2021c). Importantly, feeding 25(OH)D_3 decreased severity of mastitis (Poindexter et al., 2020) and serum 25(OH)D_3 concentrations were associated with decreased risk for retained placenta and metritis (Wisnieski et al., 2020). Besides immunity, the VDR is expressed in mammary cells and vitamin D signaling is necessary for normal mammary development (Zinser and Welsh, 2004). Ongoing research should provide more clarity on the benefits of feeding 25(OH)D_3 in closeup diets, but for the time being it is a promising nutrient for improving transition cow performance.

Another recent development for prevention of hypocalcemia is use of low P diets or inclusion of aluminosilicate to bind Ca and P in closeup diets. The idea of low P diets originated many years ago (Barton et al., 1987), but it was only recently that it has been implemented. The concept underlying this approach is based on the function of FGF23. FGF23 release from bone in response to elevated P suppresses renal Pi reabsorption and 1,25$(OH)_2D_3$ synthesis (Shimada et al., 2004). Ultimately, FGF23 counteracts the

calcitropic and phosphotropic actions of PTH and $1,25(OH)_2D_3$. Specific evidence in support of this concept is still lacking in cows but practically the concept is supported by evidence from experimental manipulation of dietary P. Feeding prepartum acidogenic diets with 0.21 or 0.31% dietary P increased serum Ca during the last week of gestation and first 12 h postpartum compared with feeding 0.44% dietary P (Peterson et al., 2005). Likewise, feeding 0.16% dietary P prepartum increased serum Ca on days 1, 2, and 4 postpartum compared with feeding 0.30% P (Wächter et al., 2022). Inclusion of aluminosilicate in prepartum diets effectively decreases dietary P availability and it also results in improved postpartum Ca status (Kerwin et al., 2019). Restriction of postpartum dietary P is detrimental to cows, but prepartum dietary P should be restricted to the extent that is economically and practically feasible.

The most effective and well-documented approach to prevent postpartum hypocalcemia is still the use of prepartum acidogenic diets. A meta-analysis of 42 experiments found that prepartum acidogenic diets increased postpartum DMI, milk and ECM yield, and reduced incidences of retained placenta and metritis compared with alkalogenic diets (Santos et al., 2019). Acidogenic diets are effective at increasing vitamin metabolism and Ca flux. For example, feeding a diet with DCAD of -181 mEq/kg DM increased plasma $1,25(OH)_2D_3$ and Ca concentrations in response to PTH challenge compared with a diet with DCAD of 188 mEq/kg DM (Goff et al., 2014). Feeding a diet with DCAD of -153 mEq/kg DM for 7 d also increased total tract Ca absorption by 15 g/d (18 vs. 33 g/d; 24 vs. 39 % apparent digestibility) and urinary Ca excretion by 15 g/d (2 vs. 17 g/d) compared with DCAD of 236 mEq/kg DM (Vieira-Neto et al., 2021a).

Use of low K forages, laboratory analysis of forages, and consistent mixing and feeding of diets are key to the effectiveness of prepartum acidogenic diets. Some constraints, like availability of low K forages and number of cows in closeup groups, present a challenge for proper implementation of prepartum acidogenic diets. Regardless, no other approach to managing postpartum hypocalcemia has yet to demonstrate similar health and production benefits as acidogenic prepartum diets.

Conclusion

Vitamin D is part of an intricate endocrine system that regulates Ca, P and skeletal homeostasis. The inability of dairy cows to adapt to the rapid irreversible loss of Ca at the onset of lactation results in hypocalcemia. Consequences of hypocalcemia are further complicated by inflammation and poor digestive function. Supplemental $25(OH)D_3$ provides a more effective alternative to vitamin D_3 for dairy cows. Adding $25(OH)D_3$ to closeup diets does not reduce risk of immediate postpartum hypocalcemia but it does help restore Ca faster, thereby reducing the occurrence of delayed and chronic subclinical hypocalcemia. Feeding low P and acidogenic diets prepartum has a greater impact on postpartum Ca than vitamin D supplementation but vitamin D also contributes to many other aspects of physiology, notably immunity and mammary development.

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Choline is a Methyl Donor in Dairy Cows: The Proof is in the Label

J. W. McFadden and T. L. France
Department of Animal Science
Cornell University

Introduction

Choline is a quasi-vitamin with unique metabolic fates. In the gastrointestinal tract, unprotected choline may be converted to trimethylamine by the actions of trimethylamine lyase. In turn, trimethylamine may pass into circulation and subsequently transformed into trimethylamine *N*-oxide (TMAO) in the liver by flavin monooxygenase 3. Choline absorbed at the intestines, via choline transporters, is subject to a wide array of metabolic uses. First, choline may be used for the synthesis of complex phospholipids including phosphatidylcholine (PC) and lysophosphatidylcholine (LPC). Phosphatidylcholine is a component of bile, and a cellular and lipoprotein membrane lipid synthesized by the cytidine diphosphate (CDP) choline pathway (i.e., Kennedy pathway). The initial reaction, controlled by choline kinase, produces phosphocholine. Sphingomyelin synthase utilizes ceramide and PC to produce sphingomyelin, which supports brain myelination. The Lands cycle is responsible for the conversion of PC into LPC, which includes the lipolytic enzyme lipoprotein-associated phospholipase A_2 in circulation. Lysophosphatidylcholine has received attention in humans as a bioactive lipid with immunoregulatory properties (Kabarowski, 2009). Moreover, LPC has been implicated in the endotoxin-response in lactating dairy cattle (Javaid et al., 2022). Choline may also be oxidized to betaine, a key osmoregulator, by the actions of the zinc metallo-enzyme betaine-homocysteine methyltransferase.

In humans and cows, choline and betaine have been defined as methyl donors (McFadden et al., 2020). A methyl donor helps produce *S*-adenosylmethionine (SAM), via the actions of the folate and methionine cycles. In turn, SAM is used for various methylation reactions including methyl tagging on histones and deoxyribonucleic acid. Furthermore, the methylation of phosphatidylethanolamine (PE) is controlled by phosphatidylethanolamine *N*-methyltransferase (PEMT), which transforms PE to PC via three sequential methylations using SAM. In non-ruminants, the CDP choline pathway is believed to account for 70% of PC synthesis in the liver (DeLong et al., 1999). The remaining 30% of PC produced by the actions of PEMT (DeLong et al., 1999). These percentages were determined by culturing rat primary hepatocytes with tritium-labeled choline chloride or ethanolamine hydrochloric acid followed by lipid extraction and phospholipid separation. Additional experiments by DeLong and coworkers (1999) demonstrated that the fatty acyl composition is unique for PC produced by PEMT, which has a preference for fatty acyl chains of high unsaturation (e.g., C22:6). As established in choline-deficient rats, PEMT compensates for a lack of dietary choline, which may be critical during periods of inadequate dietary choline supply such as starvation, pregnancy, or lactation (Cui and Vance, 1996).

Choline Biology and Nutrition in the Dairy Cow

In dairy cattle, ruminal degradation of unprotected choline to trimethylamine is rapid (Neill et al., 1978; Sharma and Erdman, 1989). This discovery triggered the development of rumen-protected choline chloride to prevent ruminal degradation and increase dietary choline supply to the small intestines. In situ rumen degradation and in vitro intestinal digestibility methodologies have been used to estimate metabolizable choline; albeit, such an approach has ignored choline degradation by bacterial trimethylamine lyase in the intestines and thus likely overestimates metabolizable choline supply. Moreover, studies have demonstrated lower net choline absorption at the small intestine for cows fed rumen-protected choline chloride, relative to abomasal choline chloride infusion (de Veth et al., 2016), and demonstrated marked increases in plasma TMAO concentrations following the a pulse dose of rumen-protected choline chloride to the rumen (France et al., 2022). Regardless, a plethora of studies have demonstrated positive effects of rumen-protected choline feeding on milk production and composition, measures of hepatic health, disease prevention, and fertility (Humer et al., 2019; Arshad et al., 2020), which suggests the delivery of choline to the intestines for absorption and use by the cow.

In the dairy cow, identifying choline as a methyl donor has been debated. Chandler and White (2017) concluded that increasing the supply of choline to bovine neonatal hepatocytes may have supported methionine regeneration from the folate methyl pool; albeit, PEMT expression was reduced by choline chloride supplementation. Feeding cows rumen-protected choline has been shown to increase plasma concentrations of betaine and phosphocholine, which suggests the provision of substrate for transmethylation and activation of the CDP choline pathway, respectively (de Veth et al., 2016; France et al., 2022). However, evidence suggests that stage of lactation uniquely influences the choline metabolite response (i.e., preferred pathway activation) to dietary rumen-protected choline supplementation (de Veth et al., 2016; France et al., 2022). In dairy cow primary liver cells, gene evaluation data has suggested that transmethylation and transsulfuration are more responsive to methionine supplementation; whereas, choline supplementation preferentially actives the CDP-choline pathway (Zhou et al., 2018).

Several gaps in knowledge remained to adequately define choline biology in the pregnant and lactating dairy cow. First, direct evidence to support the ability of dietary choline to support hepatic methylation of PE was needed. Second, we lacked an understanding for the extent of post-ruminal choline degradation to trimethylamine by bacterial trimethylamine lyase, which has implications for defining metabolizable choline supply using in vitro intestinal digestibility assays. Third, our understanding of choline utilization between the CDP choline pathway and PEMT pathway during gestation and lactation required clarity. Fourth, direct evidence to support endogenous recycle of dietary choline, via bile, has received little attention. Fifth, the extent of dietary choline metabolized by the cow versus what is excreted in urine or secreted in milk required clarity. To answer these questions, the use of deuterium-labeled choline chloride is a means to trace choline and methyl group utilization in the cow.

Lessons Learned from Using Deuterium-labeled Choline Chloride in Humans

Deuterium is a stable isotope of hydrogen. Methyl-d9-choline chloride may be used to trace methyl group utilization in mammals. Such an approach allows the investigator to evaluate the partitioning of labeled choline (i.e., d9-choline) and choline metabolites with labeled methyl groups (i.e., d3- or d6-metabolites). The approach allows for assessment of choline and choline methyl group partitioning between the CDP choline pathway (i.e., d9-PC) and PEMT pathway (e.g., d3- or d6-PC), respectively. Since d6-PC is often undetectable or detected at low concentrations, the detection of d9-PC derived from PEMT activation is unlikely because of the greater concentration of unlabeled methyl groups, relative to labeled methyl groups, in the biological system. Figure 1 provides a schematic for methyl group utilization during choline metabolism.

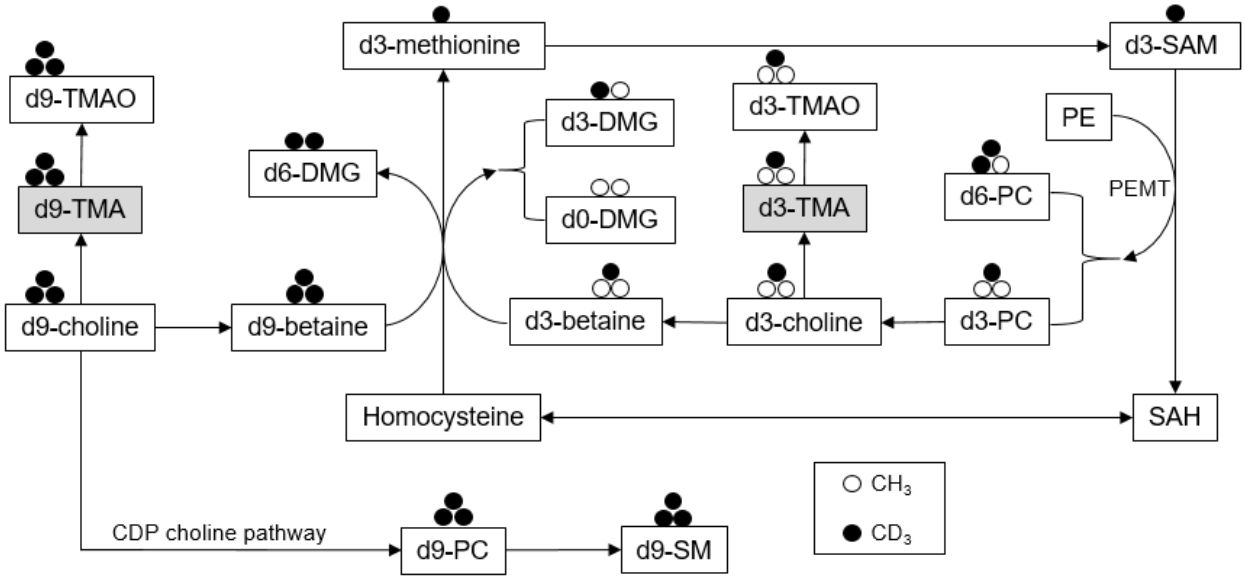

Figure 1. Deuterium-labeled methyl-d9-choline for methyl group tracing. Black circles represent deuterium-labeled methyl groups. White circles represent unlabeled methyl groups. The grey box reflects trimethylamine (TMA) produced from bacterial metabolism in the gut. Abbreviations not found in main body: DMG, dimethylglycine; SAH, *S*-adenosylhomocysteine; SM, sphingomyelin. Adapted from Yan et al. (2013) with modifications.

The use of methyl-d9-choline chloride was used in pregnant and nonpregnant women (Yan et al., 2013) and lactating women (Davenport et al., 2015) to evaluate methyl group utilization. A total of 22% or 20% of total choline intake was provided as isotopically labeled choline tracer (i.e., methyl-d9-choline chloride) for 6 wk (final 6 wk of 12 wk choline supplementation) or 10 wk, respectively. It was concluded that pregnancy increases the demand for choline, which was supported by enhanced use of choline for d9-PC and d3-PC production via the CDP choline and PEMT pathways, respectively. In nonpregnant and pregnant women, additional evidence to support the use of methyl-d9-choline methyl groups for transmethylation including increased blood enrichment of d3-SAM and d3-choline in response to increased dietary choline supplementation levels

(480 to 930 mg/d). Increasing dietary choline supplementation elevated blood enrichment of d9-betaine, suggesting enhanced choline oxidation, and d3-methionine. Yan and coworkers (2013) also observed enhanced use of PEMT-derived PC for fetal use during pregnancy. Davenport and coworkers (2015) were able to demonstrate improvements in breast milk choline supply in women that exceeded current dietary choline recommendations by increasing the production of PEMT-derived choline metabolites. However, increased choline intake also increased plasma and milk concentrations of TMAO as well as urinary TMAO yield (i.e., unlabeled and d9-TMAO).

Abomasal Choline Chloride Infusion and Methyl Group Utilization in Cows

Our objective was to evaluate the effects of abomasal choline chloride infusion on methyl group utilization in pregnant and lactating dairy cows using stable isotope methodology. Six multiparous, rumen-cannulated Holstein dairy cows (779 ± 72.0 kg of body weight) were enrolled in a longitudinal study design following transport from the Cornell Dairy Research Center (Harford, NY) to the Large Animal Research and Teaching Unit (Ithaca, NY). Cows were acclimated to the facility for 1 wk. Both pre- and postpartum diets were formulated using CNCPS v. 6.5 as implemented by AMTS.Cattle.Professional v. 4.14 (AMTS, LLC; Groton, NY). Diets were formulated to contain no supplemental rumen-protected choline chloride and deficient in methionine (< 0.96 g Met / Mcal metabolizable energy). Cows were milked twice daily at 0600 and 1700 h. Cows were fed once daily after morning milking.

Prepartum (wk -3 prior to expected due date) and postpartum (+2 wk relative to parturition) cows received a continuous abomasal infusion of unprotected choline chloride (18 g of choline chloride/d; 13.5 g of choline ion/d; Sigma-Aldrich, St. Louis, MO) for a 5-d infusion period. Methyl-d9-choline chloride (98% purity; Cambridge Isotope Laboratories, Inc., Andover, MA) replaced 20% of unlabeled choline chloride daily. Unlabeled and labeled choline chloride were dissolved in 4.1 L of water per day and infused at a rate of 170 mL/h using a pump. Cows were fed ad libitum during acclimation and between pre- and postpartum infusion periods. During the infusions, cows received their daily feed allotment as equal provisions every 4 h starting at 0 h, relative to the start of infusions.

Milk yields were recorded daily. Milk samples were collected at each milking during the last 2 days of the covariate and infusion periods. Blood samples were collected for plasma separation at 0600 h daily during each infusion period. Urinary catheters were used during the last 2 consecutive days of the infusion periods to collect total urine. Urine pH was measured at the end of each day and maintained at a pH ≤ 2 using hydrochloric acid. Liver tissue (~1 g) was biopsied using a custom fabricated trocar on the final hour of each infusion period. Milk, plasma, urine, and liver samples were frozen at -80°C until analysis.

Choline, betaine, dimethylglycine, methionine, glycerophosphorylcholine, phosphocholine, PC, LPC, and sphingomyelin were extracted from all biological

samples and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Koc et al. (2002) with modifications described by Yan et al. (2014). Trimethylamine *N*-oxide and trimethylamine were measured using the methodology described by Wang et al. (2011) with modifications by Yan et al. (2012). Enrichment percentages of isotopically labeled choline metabolites in plasma, liver, milk, and urine were calculated in accordance with Chew et al. (2011) and Yan et al. (2014).

We confirm that the abomasal infusion of choline chloride at 13.5 g/d with 20% enrichment with methyl-d9-choline chloride was adequate to achieve a steady state plasma concentration of d9-choline by d 5 of infusion. Bacterial degradation of choline to trimethylamine and TMAO was evident with plasma enrichments of d9-trimethylamine (2.78 and 4.01% during pregnancy and lactation, respectively; $P = 0.21$) and d9-TMAO (13.2 and 4.87% during pregnancy and lactation, respectively; $P < 0.05$). We observed evidence for choline oxidation to betaine by detecting plasma enrichment of d9-betaine (9.83 and 12.1% during pregnancy and lactation, respectively; $P < 0.05$) and d6-dimethylglycine (33.3 and 27.2% during pregnancy and lactation, respectively, $P = 0.46$). We detected evidence for choline utilization by the CDP choline pathway including enrichment of plasma d9-PC (4.85 and 5.71% during pregnancy and lactation, respectively), liver d9-phosphocholine (5.43 and 6.38% during pregnancy and lactation, respectively) and d9-PC (5.40 and 6.28% during pregnancy and lactation, respectively), and milk d9-phosphocholine and d9-PC (4.82 and 4.18% during lactation, respectively). We also provide direct evidence for the use of choline methyl donors for hepatic methylation of PE including enrichment of plasma d3-PC (0.22 and 0.16% during pregnancy and lactation, respectively; $P = 0.33$) and d3-choline (0.07 and 0.14% during pregnancy and lactation, respectively; $P = 0.08$), and liver d3-PC (0.40 and 0.41% during pregnancy and lactation, respectively; $P = 0.96$) and d3-choline (0.24 and 0.25% during pregnancy and lactation, respectively; $P = 0.87$), and milk d3-phosphocholine and d3-choline (0.15 and 0.15% during lactation, respectively). Evidence for endogenous recycling of choline via bile was also detected (e.g., enrichment of plasma d3-trimethylamine at 6.23 and 9.28% during pregnancy and lactation, respectively; $P = 0.19$).

The presentation will provide a complete evaluation of plasma, liver, milk, and urinary isotope enrichments of choline and choline metabolites. We will highlight liver enrichment ratios to consider enzyme activation or deactivation. We will also provide estimates for the extent of post-ruminal choline degradation to trimethylamine, the amount of abomasal-infused choline chloride used for milk choline and choline metabolite production, and the amount of choline and choline metabolites excreted in urine or secreted in milk in response to abomasal choline chloride infusion.

Conclusion

The use if stable isotope methodology has provided us definitive evidence that choline is a methyl donor in the pregnant and lactating dairy cow. In addition, we provide evidence for choline utilization by the CDP choline and PEMT pathways, and endogenous recycling of absorbed choline via bile. Our findings also provide insight into the degree of post-ruminal choline degradation to trimethylamine, which will emphasize the importance of correcting estimates of metabolizable choline when in vitro systems are applied. We also demonstrate that the use of methyl-d9-choline chloride and mass spectrometry are means to assess choline bioavailability in the cow.

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Nutritional Mitigation of Heat Stress-Induced Leaky Gut: The Role of DCAD and Dietary Buffer

C. Bertens[1], C. Stoffel[2], and G.B. Penner[1]
[1]Department of Animal and Poultry Science, University of Saskatchewan
[2]Papillon Agriculture Company, Easton, MD

Introduction

Heat stress (**HS**) in lactating dairy cows is a multifactorial disorder that can lead to reductions in milk yield of up to 40% (Tao et al., 2018) and negatively impacts reproductive success thereby imposing a large financial burden at the farm level. Even dairy cattle in temperate climates, including northern regions of the United States and Canada experience mild to moderate HS, and it can be challenging to maintain milk production and performance during summer months (Ominski et al., 2002). While barn design and implementation of heat abatement systems largely dictate severity of heat stress exposure, nutritional strategies to mitigate heat stress may also be applied (Baumgard and Rhoads, 2009).

In response to elevated heat load, cows adapt by reducing DMI, increasing water intake, increasing respiration rate, and increasing sweating (Bernabucci et al., 2010). Increased respiration rate has been a particular focus and reduces pCO_2 in blood inducing respiratory alkalosis. Secondary to respiratory alkalosis is a compensatory metabolic acidosis as urinary HCO_3 excretion increases (Silanikove, 2000; Kadzere et al., 2002). Sweating and panting contribute to the loss of electrolytes including Na^+, K^+, Ca^{2+}, Mg^{2+}, P, Cl^-, HCO^-_3, and HPO^{4-} (Bailey and Balch, 1961) and altered concentrations along with reduced ruminal motility have been suggested to increase risk for ruminal acidosis during heat stress exposure (Burhans et al., 2022).

To offset losses of electrolytes the DCAD concentration of diets may be increased during months with risk for heat stress. Common dietary ingredients to increased DCAD include $NaHCO_3$, $KHCO_3$, and K_2CO_3 (West et al., 1987). Increasing DCAD has been reported to help cattle cope with heat stress as indicated by maintenance of blood acid-base balance (Wildman et al., 2007), greater DMI (Hu and Murphy, 2004), and greater water intake (Tucker et al., 1998). However, use of Na+ or K+ coupled with carbonates add an inherent confounding effect as they also provide a dietary buffer. Typically, the added buffer is viewed as being a supportive strategy to mitigate risk for ruminal acidosis co-occurring with heat stress, but this precludes confirmation if responses are driven through cations or dietary buffer.

The loss of electrolytes reduces the buffer supply to the rumen which has been postulated to increase the risk for subacute ruminal acidosis (**SARA**) leading to gastrointestinal tract (**GIT**) barrier dysfunction (Burhans et al., 2022; Plaizier et al., 2022). Ruminal acidosis may trigger local inflammation and increased GIT permeability thereby allowing for the translocation of endotoxins and live bacteria into circulation

eliciting systemic inflammation (Liu et al., 2013; Burhans et al., 2022; Plaizier et al., 2022). In addition to ruminal hyperpermeability, compromised intestinal barrier function resulting from HS has been investigated in non-ruminant species including humans (Lim, 2018) and pigs (Pearce et al., 2012; Mayorga et al., 2020); however, there is a paucity of data for ruminants. Gastrointestinal tract permeability and resulting systemic inflammation is suspected to be the etiology of HS associated milk production losses, mortality, and morbidity (Baumgard and Rhoads, 2013; Burhans et al., 2022).

Is Na^+ Limiting in the Rumen?

Regulation of ruminal pH is complex an involves acid removal mechanisms arising from short-chain fatty acid absorption, neutralization of acid via salivary buffer, passage of acid out of the rumen, and in buffering driven by other ions such as ammonia/ammonium and phosphates (Allen, 1997; Aschenbach et al., 2011). It has been proposed that cattle experiencing heat stress may suffer from a ruminal Na deficiency that compromises short chain fatty acid (**SCFA**) absorption (Mooney, 2006) increasing susceptibility to SARA (Burhans et al., 2022). While not evaluated under heat stress conditions, a recent ex vivo study reported no evidence for Na+ to affect the uptake of acetate or butyrate (Bertens et al., 2023) supporting previous results of Sehested et al. (1999) indicating Na+ concentration did not affect flux of acetate or butyrate across the isolated ruminal epithelium.

To further test this concept, we exposed cows to moderate heat stress (temperature humidity index between 68 and 72) and evaluated short-chain fatty acid absorption using the temporarily isolated and washed reticulo-rumen technique under low (45.9 mmol) and high Na (85.3 mmol) buffer conditions (Bertens et al., 2022). In that study, altering the concentration of Na+ in the incubation buffers had no effect on the rates of SCFA absorption in vivo. Collectively, these data suggest that it is unlikely that ruminal Na+ concentration limits SCFA absorption.

Comparing DCAD and Dietary Buffer

We conducted an experiment to separate out effects arising from DCAD and dietary buffer using a 2×2 factorial treatment arrangement within a replicated (16 cows; 8 primiparous and 8 multiparous) 4×4 Latin square. Part of these data were reported in Bertens et al. (2022) with the remainder being unpublished. In this study, we altered the DCAD primarily with the inclusion or exclusion of Na-acetate and altered dietary buffer supply by including or excluding $CaMg(CO_3)_2$ (0 vs. 1% of dietary DM, MIN-AD, Papillon Agriculture Company, Easton, MD). Achieved DCAD values were 17.6 and 39.6 mEq/100 g for the low and high DCAD treatment, respectively. All cows were exposed to a temperature-humidity index (THI) averaging 73 ± 1.4 from 0600 h to 1600 h with allowance for natural night cooling from 1601 h to 0559 h achieving a mean THI of 67 ± 2.5. As evidence for heat stress conditions, cows had elevated rectal temperatures throughout the study averaging 39.1°C with no effect of DCAD, buffer, or their interaction.

In the above-mentioned study (Bertens et al., 2022), providing added buffer or increasing the DCAD did not affect DMI with an average intake of 25.1 kg/d. This result differs from several past studies where increasing DCAD stimulates DMI (Hu and Murphy, 2004; Iwaniuk and Erdman, 2015). Given the quadratic relationship between DMI and DCAD (Hu and Murphy, 2004), it is possible that the difference in DCAD was not sufficiently different to detect differences. While we could not measure water intake in this study, we did observe that cows fed greater DCAD had urine output that was 3.8 L/d greater (P =0.02) than when fed the low DCAD treatment. Increased water intake is one strategy cows can employ to mitigate heat stress as they transfer body heat to the water and excrete it as urine (McDowell et al., 1969; Bernabucci et al., 2010). Increasing DCAD with Na-acetate did not affect serum Na+ concentrations but resulted in lower concentrations of serum K (4.5 vs. 4.6 mmol/L; P = 0.02) and Cl (96.1 vs. 98.2 mmol/L; P < 0.01). Increasing dietary buffer increased serum Ca concentration (2.8 vs. 2.4 mmol/L; P = 0.04). There was an interaction between buffer inclusion and DCAD (P = 0.03) such that cows fed added buffer had greater serum HCO_3 when fed high DCAD than low DCAD, while there were no effects of DCAD when fed without added dietary buffer. Moreover, values for the low buffer treatments (low and high DCAD) were intermediate and not different to the high buffer with high DCAD and high buffer with low DCAD. Changes in the concentration of Ca with high buffer may have affected the concentration of HCO_3 according to the strong ion theory (Goff, 2018). Given the changes in serum mineral concentrations, the calculated anion gap was above 21.1 mEq/L suggesting cows were all in a very mild metabolic acidosis (Goff, 2018).

Milk yield was not affected by treatments averaging 36.9 L/d (Bertens et al., 2022). That said, providing a greater DCAD increased milk fat yield (1.53 vs. 1.50 kg/d; P = 0.03) and reduced the proportion of preformed milk fatty acids (45.5 vs. 47.4%; P < 0.01) but there were no effects on other milk components with greater DCAD or buffer inclusion. Wildman et al. (2007) reported increased milk fat with elevated DCAD using $NaHCO_3$ during heat stress in which they attributed the improvement in milk fat to enhanced ruminal buffering. Ruminal pH was not affected in the present study challenging whether DCAD directly would affect buffering. That said, we used Na-acetate to increase DCAD and a previous study using slightly higher inclusion rates of Na-acetate reported an increase in milk fat and reduced preformed fatty acids (Urrutia et al., 2019). As such, it is not possible to attribute milk fat responses directly to DCAD in the present study.

Ruminal pH

It is commonly reported that cows exposed to heat stress experience ruminal acidosis due to loss of electrolytes and consequently reduced salivary buffer supply (Burhans et al., 2022) along with altered sorting behavior (Baumgard and Rhoads, 2009), and feeding patters (Frazzi et al., 2000). Although we could not evaluate the effect of heat stress on ruminal pH as all cows were exposed to heat stress conditions, there were no effects of DCAD or dietary buffer inclusion on ruminal pH with mean ruminal pH averaging 6.39 (Bertens et al., 2022). While DCAD alone would not be expected to alter pH, the efficacy of dietary buffers to act within the rumen relies on pH

to allow for complete or partial solubilization (Le Ruyet and Tucker, 1992). For $CaMg(CO_3)_2$, reductions in pH increase solubilization (Altland and Jeong, 2016). In the present study, mean ruminal pH was 6.39 and minimum pH was 5.85 which likely resulted in only partial ruminal solubilization of Ca and Mg $(CO_3)_2$. Hence, the chemical properties of $CaMg(CO_3)_2$ most likely explains the undetected effect of buffer to modulate ruminal pH; however, the increase in serum Ca with greater buffer inclusion suggests that, at least in the total tract, Ca supply was improved with buffer supply. These findings are supported by Crawford et al. (2008) in which there was limited effect of $Ca(CO_3)_2$ on ruminal pH in growing yearling steers (mean ruminal pH of 6.0). Razzaghi et al. (2021) evaluated the effect of $NaHCO_3$, MgO, and $CaMg(CO_3)_2$ on ruminal pH, measured continuously, in lactating dairy cattle fed diets containing 34% starch. They found no difference in mean ruminal pH (5.76 ± 0.04) but saw increases in maximum pH and minimum pH with all three buffer supplements. In addition, they found that the area below 5.8 was reduced with all buffer supplementation when compared to the control, with the greatest effects seen for $NaHCO_3$ followed by lesser but similar effects seen for MgO and $CaMg(CO_3)_2$.

Permeability of the Gastrointestinal Tract

There has been a growing body of evidence supporting that heat stress increases permeability of the gastrointestinal tract. In fact, direct effects of heat stress have been reported to compromise gastrointestinal barrier function in ruminants (Koch et al., 2019), rodents (Lambert et al., 2002), pigs (Pearce et al., 2012), and humans (Lim, 2018). Therefore, heat stress-induced leaky gut may result independently of ruminal acidosis. As an approach to separate ruminal and post-ruminal permeability, we developed a novel technique utilizing ruminally infused Cr-EDTA to indicate total tract permeability (Zhang et al., 2013) along with a simultaneous abomasal infusion of Co-EDTA to indicate post-ruminal permeability (Bertens et al., 2022). In the heat stress study evaluating buffer and DCAD describe above, we detected tendency ($P = 0.098$) for added $CaMg(CO_3)_2$ to reduce the amount Cr-EDTA recovered in urine and a reduction in the recovery of Co-EDTA ($P < 0.01$). These data suggest that that dietary buffer may help improve barrier function of the gastrointestinal tract largely by reducing post-ruminal permeability. These data are supported by a recent study showing that heat stress alters intestinal barrier function in the jejunum along with changes in the microbial community structure in the colon. Other studies in our laboratory have further confirmed that intestinal regions may be more sensitive to barrier dysfunction than ruminal regions (Penner et al., 2014; Lambert et al., 2023) supporting the results of Bertens et al. (2022).

While we cannot confirm the mechanisms involved in promoting barrier function with added dietary buffer (Bertens et al., 2022), the potential for $CaMg(CO_3)_2$ to solubilize in the abomasum may lead to potential buffering effects more distally in the gastrointestinal tract. In agreement, Rauch et al. (2012) utilized an in vitro technique with $CaMg(CO_3)_2$ and reported no change in ruminal pH. However, in the same paper, the authors tested the effects of different buffer supplements fed to lactating cows under thermoneutral conditions and demonstrated elevated fecal pH in cows supplemented

with $CaMg(CO_3)_2$ when compared to cows fed $NaHCO_3$ and control diets. It may be possible that dietary buffers that solubilize post-ruminally may help regulate intestinal fermentation and regulation of the intestinal barrier. Future research is needed to confirm these findings and to evaluate potential mechanisms for such an effect.

Conclusion

In summary, it does not appear that ruminal Na will limit SCFA absorption or potential effects on ruminal pH. Provision of $CaMg(CO_3)_2$ as a dietary buffer and altering DCAD do not interact to affect DMI, ruminal fermentation, and GIT permeability in lactating dairy cattle exposed to mild heat stress. However, elevated DCAD as affected by Na-acetate increased urine output and increased milk fat yield. Despite the lack of ruminal acidosis in the present study, $CaMg(CO_3)_2$ reduced intestinal permeability and tended to reduce total tract permeability while DCAD had no effect. These findings highlight the pitfalls of the current literature emphasizing ruminal permeability associated with ruminal acidosis without sufficient acknowledgement for post-ruminal permeability. Consequently, these findings may extend value of dietary buffer beyond the rumen and help to provide new information on the individual contributions of dietary buffer and DCAD under mild heat stress conditions.

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Relationships of Blood-based Indices of Liver Function During the Transition Period with Performance and Health in Dairy Cattle

T. M. Nelson
Department of Animal Science
Cornell University

Introduction

All cows undergo physiological and metabolic changes in the periparturient period that help the cow adapt to lactation (Trevisi and Minuti, 2018). These changes can promote inflammation and when they are dramatic or prolonged, can lead to poor health status and performance (Bertoni and Trevisi, 2013). A diagnostic system using blood metabolites was developed by Bertoni and Trevisi (2013) to determine health status. The liver functionality index (LFI) however, with multiple timepoint sampling limited its potential for field use, and a single time point early in lactation would have more practical use in the field. The objective was to compare the proven liver functionality index to a novel liver health index (LHI) (Gallagher et al., 2019) and characterize relationships of liver health with transition period outcomes.

Results

Cows that had high LFI and LHI tended to have higher milk yield in the first 12 weeks postpartum than cows with low LFI and LHI (Figures 1 and 2, respectively). Those cows with high LFI and LHI also both had significantly lower concentrations of haptoglobin (Hp) (Figures 3 and 4, respectively), as well as have lower concentrations of non-esterified fatty acids (NEFA) compared to cows with low LFI and LHI (Figures 5 and 6, respectively) in the first two weeks postpartum.

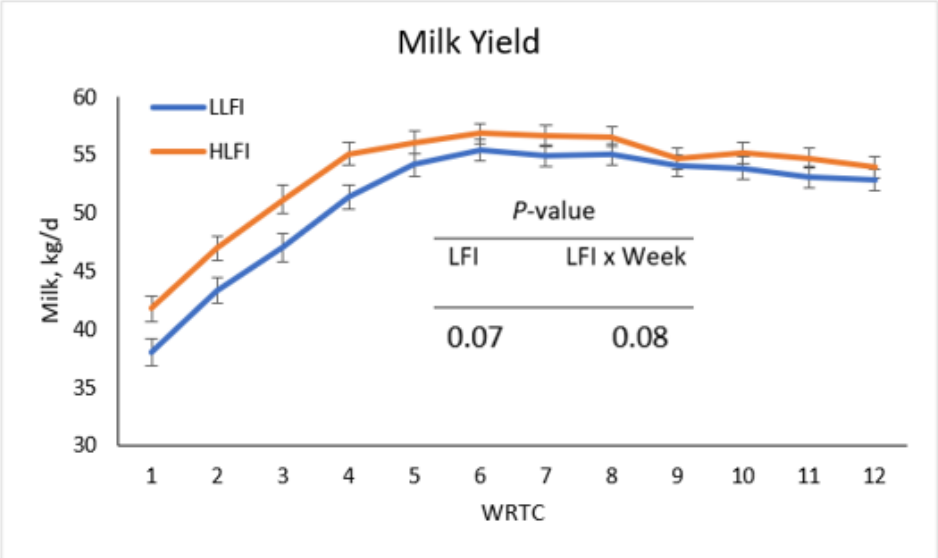

Figure 1. Milk yield of low and high LFI in the first 12 weeks postpartum.

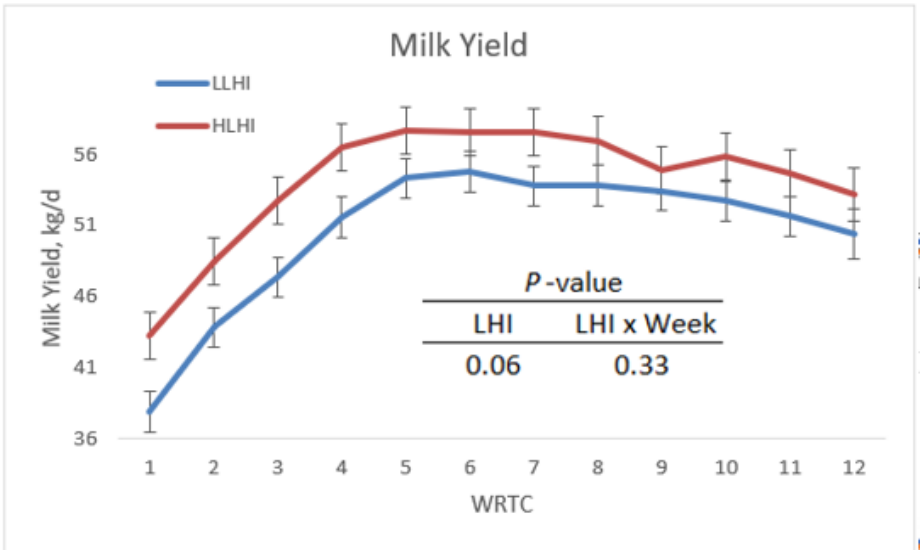

Figure 2. Milk yield of low and high LHI in the first 12 weeks postpartum.

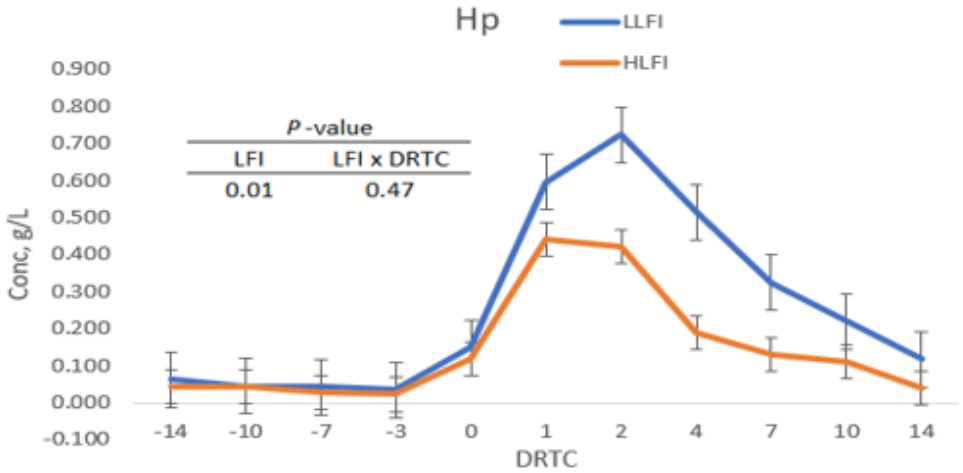

Figure 3. Haptoglobin concentrations of low and high LFI in the transition period.

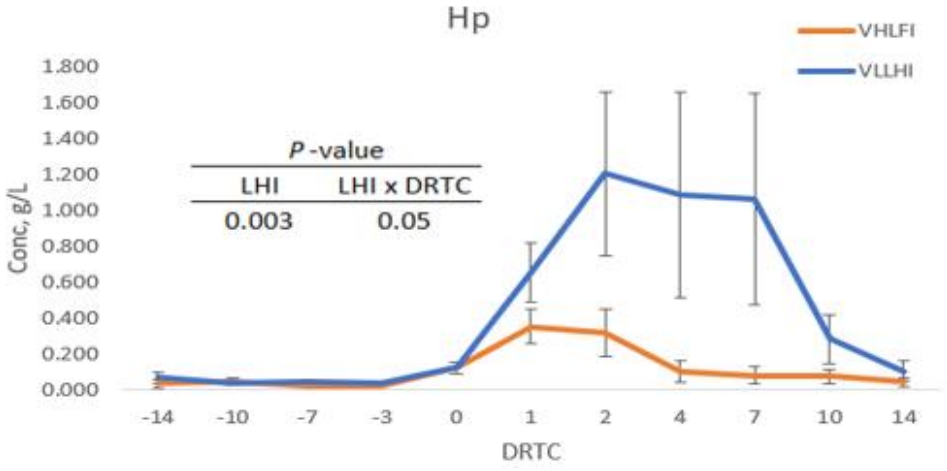

Figure 4. Haptoglobin concentrations of low and high LHI in the transition period.

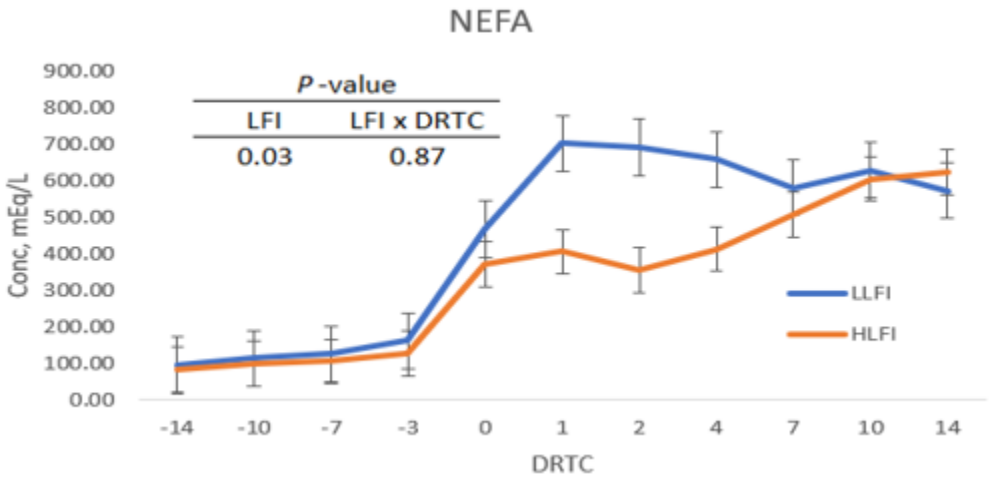

Figure 5. NEFA concentrations of low and high LFI in the transition period.

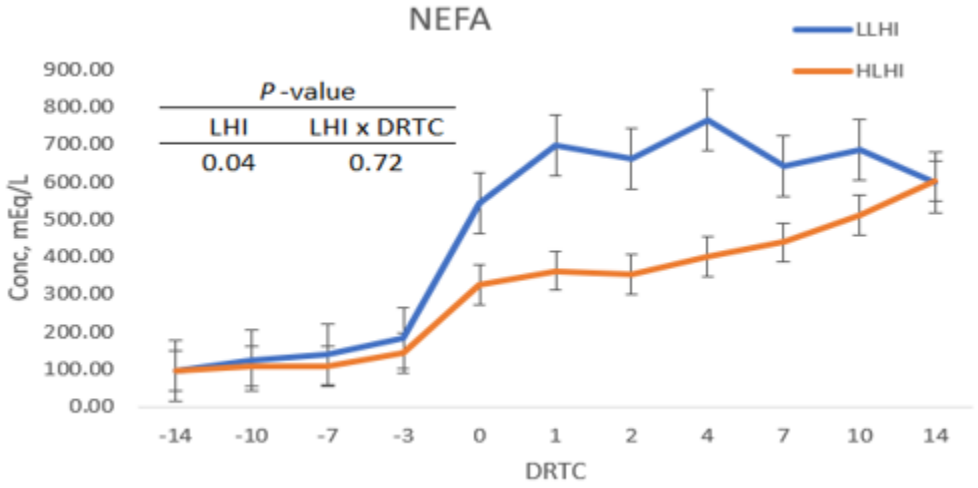

Figure 6. NEFA concentrations of low and high LHI in the transition period.

Summary and Implications

Overall LHI can be a more practical measure of potential performance than LFI due to less sampling and earlier determination in the lactation. Periodically taking samples to benchmark transition period would be recommended to assess the overall liver health of a herd. We still need to establish a reference to compare results between herds and regions. It can be a simple way for progressive producers and advisors to analyze their transition performance overall.

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Quantifying the Extent and Impact of Mixing Accuracy on Dairy Farms

M. R. Villalobos-Barquero, J. A. Barrientos-Blanco, L. E. Moraes and K. F. Reed
Department of Animal Science
Cornell University

Introduction

Feed efficiency is one of the most important factors to consider in farm production and the accuracy of feed delivery is an important component of farm feed efficiency. It is well known that the diet delivered to the cows can differ from the one that is formulated. Errors in the amounts of feeds included in a diet can increase variability in the diet composition. Frequent or large changes in diet composition can compromise the fulfillment of the nutrient requirements and therefore affect the cow's performance (Sova et al., 2014).

Methodology

Feed delivery records from six dairy commercial farms were collected during the winter of 2020 and summer of 2022. Each of the datasets was subjected to a cleaning process which included standardizing ingredient names and adding the pen count, batch ID, and laboratory analysis to the feed delivery datasets. We then calculated the error in DM delivered for each ingredient by taking the absolute value of the difference between the actual amount of the feeds delivered to each pen and the targeted amount. In addition, we identified outliers, classified them into systematic errors or real errors, amended or removed systematic errors, and summarized the cleaned datasets by pen, feed, and farm to quantify the accuracy of feed delivery across these factors.

Preliminary Results

After analyzing the six farms we found that 1.10% of the data contained systematic errors. The main systematic errors found were due to recording errors during a transition of ingredients, time offsets when the target levels were adjusted, ingredient exhaustion, and typing or software mistakes. The errors observed in Figure 1 between the target and the actual delivered corn silage (lbs DM) do not include systematic errors and illustrate an example of real errors in feed delivery that impact the accuracy of the diet mixing process.

Averaged across all 6 farms, the delivery errors for lactating cow pens showed differences between the ingredients (Figure 2). Corn meal had the largest median error (18 lbs DM) even though the average inclusion rate was relatively small at 11.9%. This was followed by corn silage with a median error of (17 lbs DM) which had a much higher inclusion rate of 30.7%. These were followed by the premix (14 lbs DM), haylage (12 lbs DM), soybean meal (7 lbs DM), and whey (2 lbs DM) which had respective inclusion rates of 19.4%, 11.4%, 3.5%, and 1.5%. Except for corn meal, the median errors appear to decrease

with the ingredient's inclusion rate. The disproportionate mixing errors with respect to the inclusion rate seen for corn meal represent an opportunity for improving the feed delivery accuracy of this feed and therefore the IOFC of dairy farms. Further investigation into the causes of these errors will help identify specific recommendations to reduce mixing errors.

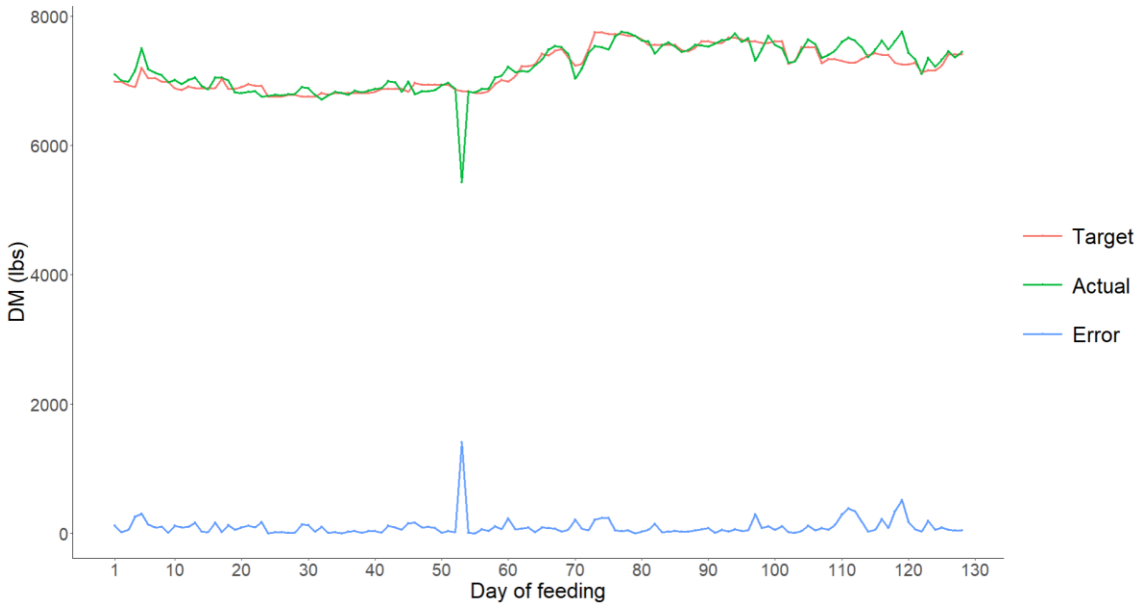

Figure 1. Difference between the target and the actual DM of corn silage delivered to the cows.

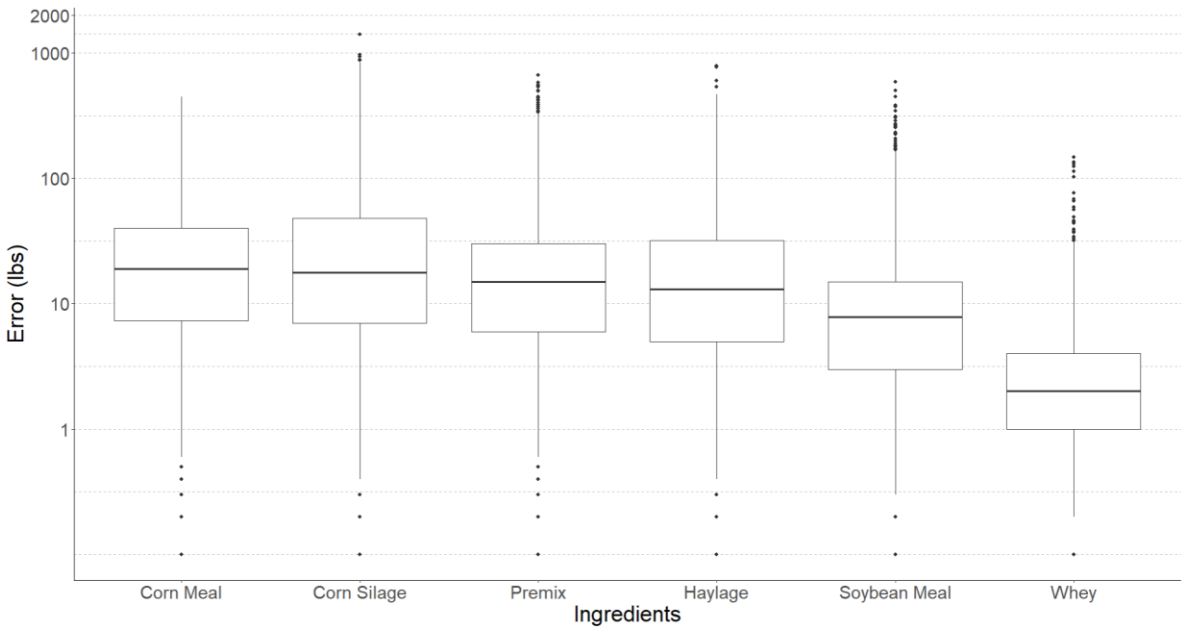

Figure 2. Boxplots of the absolute value of the error in mixing feed ingredients (DM lbs) on a log scale fed to lactating cow pens from six dairy farms over ~6 months.

Take Home Message

The systematic errors found on the datasets show that there is still space for improving methods for feed mixing data collection and cleaning to increase the utility of this data. Our preliminary analysis of the true errors in feed mixing quantified the impact of feeding accuracy on dairy farms and while median errors were relatively small, the data is highly skewed with large outliers that could still represent a false data record despite our rigorous data cleaning method. Feed delivery and inventory software developers and farmers should work together to make the data collection process more accurate and inferences from the data more accessible.

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Pre- and Postpartum Metabolizable Protein Supply Alters Performance of Multiparous Holstein Cows

T. A. Westhoff[1], T. R. Overton[2], M. E. Van Amburgh[2], J. N. Tikofsky[3], S. Mann[1]
[1]Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, NY
[2]Department of Animal Science, Cornell University, Ithaca, NY
[3]Purina Animal Nutrition, Gray Summit, MO

Introduction

Inadequate nutrient intake together with an increased nutrient demand to support fetal and mammary development as well as colostrum and milk synthesis drives transition cows into a negative energy and protein balance (Bell et al., 1995, Bell, 1995, Mann et al., 2016). Metabolizable protein (MP) requirements for multiparous, close-up dry cows were estimated at approximately 800 g/d (Husnain and Santos, 2019, NASEM, 2021). However, since the current estimate does not include the amino acid requirement for mammary growth and colostrum synthesis, prepartum MP supply might affect production of high-quality colostrum as well as early lactation milk production. Several authors have reported a positive early lactation milk production response when supplementing methionine and lysine (Batistel et al., 2017, Fehlberg et al., 2020) or increasing total prepartum MP supply (Farahani et al., 2017, Farahani et al., 2019). Milk yield during the first three weeks of lactation increased 3.4 ± 0.9 kg/d (7.5 ± 2.0 lb/d) when increasing prepartum MP supply from 849 to 1,200 g/d, but milk yield was not further increased when the prepartum diet supplied 1,387 g of MP/d (Farahani et al., 2017). Further increasing prepartum MP supply from 1,264 to 1,681 g/d resulted in a 3.7 ± 1.2 kg/d (8.2 ± 2.6 lb/d) lower milk yield from 1 to 12 wk (Zang et al., 2022). Moreover, Farahani et al., 2019 recently reported an interaction between pre- and postpartum MP supply suggesting milk yield during early lactation might depend on the supply of MP provided during both the close-up and fresh period.

During early lactation, mature Holstein cows producing a milk yield of 53 kg/d have an estimated MP requirement of 2,802 g/d. Numerous authors have demonstrated that cows respond to an increased postpartum MP supply with increased milk production (Larsen et al., 2014, Carder and Weiss, 2017, Tebbe and Weiss, 2021). Recently, Zang et al. (2021) observed a 2.5 ± 1.1 kg/d (5.5 ± 2.4 lb/d) increase in milk yield during the first three weeks of lactation when increasing MP supply from 2,227 to 2,513 g/d. However, the optimum supply of MP during the pre- and postpartum period remains unknown. Further, it is unclear whether the performance effect of altering MP supply fed during the close-up, fresh, or both periods persist beyond the treatment period. Identification of the optimum MP feeding strategy during the transition period might improve productive efficiency and increase farm profitability. As such, the objective of this study was to compare the effect of four MP feeding strategies on lactation performance, colostrum production, and the metabolic response to lactation.

Experimental design

Multiparous Holstein cows (n = 96) were enrolled in a randomized block design at the Cornell University Ruminant Center from May to November 2021. All animals were moved from a far-off dry cow pen to individual tie stalls between 35 and 42 d before expected calving and fed a far-off diet. At 28 d before expected calving, cows were blocked by calving date and balanced for parity and previous lactation 305-d mature equivalent milk production. Animals were randomly assigned within block to 1 of 4 dietary treatments groups consisting of a combination of a pre- and postpartum diet (Figure 1). The close-up TMR was formulated to contain either a control (C; 85 g of MP/kg DM) or high (H; 113 g of MP/kg DM) level of MP. Both prepartum diets were formulated to supply methionine (Met) and lysine (Lys) at 1.24 and 3.84 g/Mcal of metabolizable energy (ME), respectively. From calving to 21 days in milk (DIM), cows were fed a postpartum TMR formulated to contain either a control (C; 104 g of MP/kg DM) or high (H; 131 g of MP/kg DM) level of MP. Postpartum diets were formulated to supply Met and Lys at 1.15 and 3.16 g/Mcal of ME in both groups, respectively. The combination of a pre- and postpartum diet resulted in 4 treatment groups: 1) control-control (**CC**), 2) control-high (**CH**), 3) high-control (**HC**), and 4) high-high (**HH**), respectively. Cows were fed a common lactation diet from 22 to 42 DIM. Treatment diets were formulated using the Cornell Net Carbohydrate and Protein System v. 6.5.5 (AMTS.Cattle.Professional v. 4.17.0.0; AMTS LLC; Table 1; Van Amburgh et al., 2015).

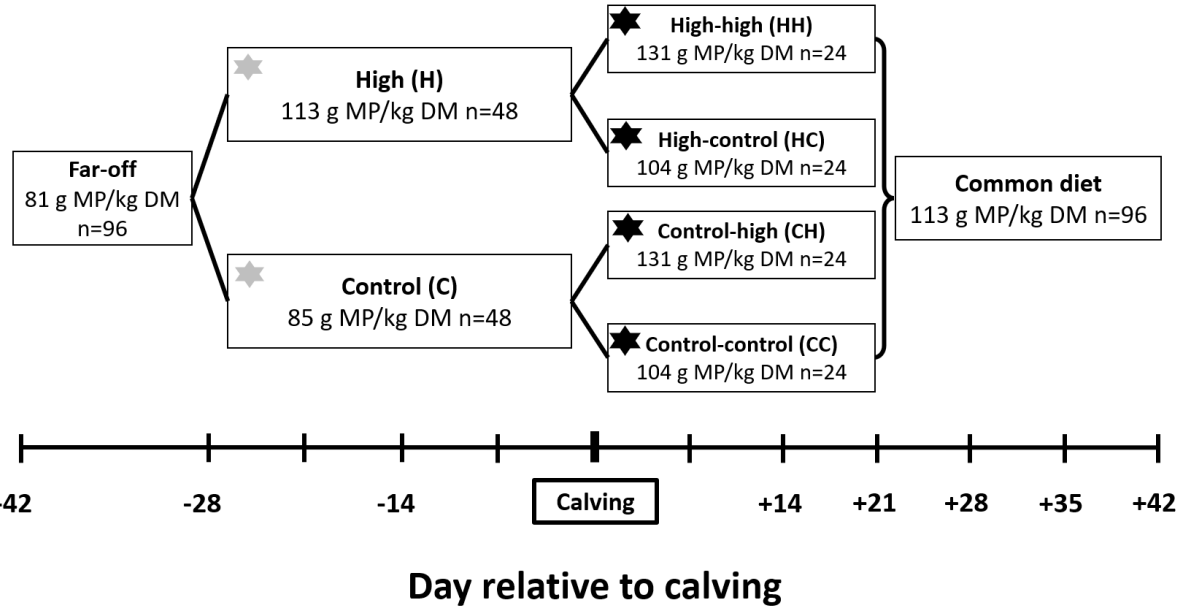

Figure 1. Schematic of treatment assignment.

Individual daily feed intake was recorded and DMI was calculated using the diet DM percentage collected weekly. Weekly, cows were weighed and BCS was determined by one investigator using a 5-point scale with 0.25-point increments (Edmonson et al.,

1989). At calving, colostrum yield and Brix percentage were recorded by farm personnel. Cows were milked three times daily and milk yields were recorded until 42 DIM. Milk samples were collected once weekly for three consecutive milkings and submitted to a commercial laboratory (Dairy One Cooperative Inc.) for analysis of fat, true protein, lactose, total solids, and milk urea nitrogen (MUN) by Fourier Transform Infrared Spectroscopy (method 972.160; AOAC International, 2012). Metabolizable protein supply and balance as well as metabolizable energy balance were estimated weekly for each cow in AMTS.Cattle.Professional v. 4.17.0.0 using the calf birth weight, DCC, BW, and weekly DMI for prepartum estimates, and DIM, BW, weekly DMI, as well as milk yield, and milk composition for all postpartum estimates.

Table 1. Ingredient composition of diets.

	Treatment diets[1]				
	Prepartum		Postpartum		
Ingredient, % of DM	Control	High	Control	High	Lactation[2]
Conventional corn silage	45	45	-	-	37.1
BMR corn silage[3]	-	-	37.3	37.3	12.1
Haylage	-	-	17.8	17.8	12.5
Wheat straw	26.7	26.7	4.7	4.7	-
Corn grain	-	-	17.2	17.2	18.6
Grain mix[4]	-	-	-	-	10.8
Soybean meal solvent	3.5	3.3	3.9	0.9	8.6
Canola meal solvent	1.8	1.5	3.8	1.2	-
Amino Plus[5]	0.7	11.0	1.0	11.4	-
ProvAAl Lysine[6]	0.9	2.2	0.60	2.20	-
Smartamine M[7]	0.13	0.04	0.17	0.10	-
USA Lysine[8]	0.45	-	0.41	0.02	-
Citrus pulp dry	-	-	2.70	1.00	-
Soybean hulls ground	10.4	1.0	3.70	-	-
Wheat midds	2.9	1.2	1.6	0.90	-
Energy Booster 100[9]	-	-	1.5	1.6	-
Sodium bicarbonate	-	-	0.76	0.70	-
Salt white	0.21	0.19	0.62	0.65	-
MIN-AD[10]	0.89	0.86	-	-	-
Magnesium oxide	-	-	0.33	0.34	-
Magnesium sulfate	0.89	0.86	-	-	-
Calcium carbonate	1.83	2.23	1.33	1.42	-
Calcium sulfate dihydrate	0.78	0.10	-	-	-
Dicalcium phosphate	0.75	0.48	-	-	-
Vitamin E mix[11]	-	-	0.01	0.01	-
Monensin[12]	0.02	0.02	0.01	0.01	-
Choline[13]	0.43	0.41	0.30	0.29	-
Animate[14]	1.27	2.48	-	-	-
Vitamin/mineral mix[15]	0.45	0.43	-	-	-
Vitamin/mineral mix[16]	-	-	0.26	0.26	-

[1]Prepartum TMRs, fed starting at 28 d before expected calving, were formulated to contain methionine and lysine at 1.24 and 3.84 g/Mcal metabolizable energy (ME), respectively and supply a control (C; 85 g MP/kg DM) or high (H; 113 g MP/kg DM) level of estimated metabolizable protein (MP). Postpartum TMRs, fed from 0 to 21 DIM, were formulated to contain methionine and lysine at 1.15 and 3.16 g/Mcal ME, respectively and supply control (C; 104 g MP/kg DM) or high (H; 131 g MP/kg DM) level of estimated MP.

[2]From 22 to 42 DIM, cows were fed a lactation diet that contained methionine and lysine at 1.01 and 2.88 g/Mcal ME, respectively and supplied 113 g of estimated MP/kg DM.

[3]Brown mid-rib corn silage

[4]Contains 28.7% Amino Plus (Ag Processing Inc.), 13.1% blood meal, 11.8% sodium sesquicarbonate, 9.4% chocolate dairy mix, 9.0% soybean hulls, 7.3% calcium carbonate, 4.2% bypass fat (Cargill Animal Nutrition), 3.5% salt, 3.0% urea, 2.7% Celmanax SCP (Arm and Hammer), 1.8% mono dicalcium phosphate, 1.7% magnesium oxide 54%, 1.7% potassium magnesium sulfate, 0.50% Smartamine M (Adisseo), 0.5% Diamond V XPC (Diamond V North America), 0.40% selenium 0.06%, 0.26% Zinpro 5 (Zinpro Corp.), 0.17% Dairy ADE (Contains 30,073 kIU/kg vitamin A, 5,783 kIU/kg vitamin D, and 92,534 IU/kg vitamin E; Cargill Animal Nutrition), 0.161% trace mineral mix (Contains 2.959% Ca, 17.503% S, 0.657% Cl, 0.231% Mg, 0.141% Na, 0.128% K, 160,210.53 mg/kg Zn, 142,105.26 mg/kg Mn, 23,684.21 mg/kg Cu, 3,526.32 mg/kg Co, 3,157.90 mg/kg I, and 196.84 mg/kg Fe; Cargill Animal Nutrition), 0.057% monensin (Monovet 90; Huvepharma), 0.05% Vitamin E (Contains 510,750 IU/kg), 0.009% copper sulfate.

[5]Heat-treated soybean meal (Ag Processing Inc.)

[6]Protein supplement (Perdue AgriBusiness)

[7]Methionine protected with pH sensitive coating (Adisseo)

[8]Rumen bypass lysine (Kemin Industries Inc.)

[9]Prilled fatty acids (Milk Specialties Co.)

[10]Magnesium limestone (Papillon Agricultural Company)

[11]Contains 18.6% Ca, 0.61% Mg, and 500,444.2 ppm Vitamin E

[12]Rumensin 90 (Elanco Animal Health)

[13]Reashure (Balchem Co.)

[14]Anionic supplement (Phibro Animal Health Corp.)

[15]Contains 26.38% Ca, 0.12% P, 0.42% Mg, 0.21% K, 3.17% S, 275 ppm Fe, 17,569 ppm Zn, 1,727 ppm Cu, 5,688 ppm Mn, 55 ppm Se, 97.3 ppm Co, 98.18 ppm I, 2,341.71 kIU/kg vitamin A, 566.68 kIU/kg vitamin D, 22,658.58 kIU/kg vitamin E.

[16]Contains 28.16% Ca, 0.33% Mg, 0.09% K, 2.32% S, 870 ppm Fe, 18,235 ppm Zn, 3,291 ppm Cu, 15,995 ppm Mn, 132 ppm Se, 550 ppm Co, 390 ppm I, 1,984.5 kIU/kg vitamin A, 551.25 kIU/kg vitamin D, 9,481.5 kIU/kg vitamin E.

Blood samples were collected twice weekly from calving to 28 DIM, and once weekly from 29 to 42 DIM. Plasma concentrations of ß-hydroxybutyrate (BHB) were measured with samples warmed to 37 ºC using a Precision Xtra point-of-care device (Abbott). Samples obtained from 3 to 10 DIM with BHB ≥ 1.2 mmol/L were defined as an event of hyperketonemia (McArt et al., 2013). Plasma concentrations of nonesterified fatty acids (NEFA) were determined in duplicate by enzymatic colorimetric analysis (HR Series NEFA-HR (2), Wako Life Sciences).

The longissimus dorsi muscle diameter and subcutaneous backfat thickness were determined by ultrasound at -30, -14, -7, 7, 21, and 40 d relative. All measurements were performed on the right side of the animal using a 5-9 MHz broadband linear transducer on a portable ultrasound (IBEX PRO; E.I. Medical Imaging). The skin near the loin and thurl region were brushed and 70% alcohol was applied as a coupling agent. Longissimus dorsi diameter was measured perpendicular to the spine as the largest diameter between

muscular fascial layers at the fourth transverse process and backfat was measured approximately 10 cm caudal of the tuber coxae as the distance between the profound fascia above the gluteus medius and the surface, excluding the measurement of the skin. The hair at the location of the ultrasound transducer during the first timepoint was clipped to ensure repeated placement at subsequent timepoints. Each measurement was performed in triplicate and averaged for analysis.

Statistical analysis was performed in SAS v. 9.4 (SAS Institute Inc.) for three separate periods (-28 to 0 days relative to calving, 1 to 21 DIM, and 22 to 42 DIM) with the exception that ultrasound data was analyzed separately pre- and postpartum. Mixed effects ANOVA were conducted in PROC MIXED to explore differences in outcome variables not repeated over time. Repeated measures ANOVA were performed for outcome variables repeated over time using PROC MIXED with the fixed effects of treatment, time, parity (2 vs. ≥ 3), and the interaction of treatment and time. The models included the random effect of enrollment block and repeated effect of time with the subject of cow. Baseline covariate measurements were included in all models when available. Tukey's post hoc test was used to adjust for multiple comparisons.

Results

Diet composition

The chemically analyzed composition of the diets is in Table 2. Model predicted MP concentration was 87 and 115 g/kg DM for the prepartum C and H diets and 101 and 127 g/kg DM for the postpartum C and H diets, respectively. Estimated rumen degradable protein increased approximately 1.5 % of DM in the prepartum H compared to the prepartum C diets. During the postpartum period, concentrations of rumen degradable protein were estimated at approximately 9.0 % of DM for the C and H diets, respectively. Estimated rumen undegradable protein increased from 3.7 to 7.1 % of DM and 5.0 to 8.3 % of DM in the C compared to H diets fed pre- and postpartum, respectively.

Prepartum period

Results from the prepartum period are in Table 3. Dry matter intake (% of BW) did not differ by treatment ($P = 0.29$) but feeding the H diet resulted in a greater MP supply compared to C (1,606 ± 27 vs. 1,180 ± 27 g of MP/d; $P < 0.01$), respectively. Cows fed a greater MP supply had a larger prepartum BW gain (4.78 ± 0.45 vs. 3.21 ± 0.45 % of BW; $P < 0.01$) compared to cows fed the C diet, yet BCS ($P = 0.76$), longissimus dorsi diameter (C: 42.8 ± 0.5; H: 43.1 ± 0.5 mm; $P = 0.73$), and backfat thickness (C: 5.7 ± 0.3; H: 6.2 ± 0.3 mm; $P = 0.15$) did not differ by treatment. In agreement with previously published work (Farahani et al., 2017, Farahani et al., 2019, Akhtar et al., 2022), prepartum MP supply did not affect colostrum yield or Brix percentage ($P \geq 0.76$) in the current study.

Table 2. Nutrient composition of diets.

Component[3] (mean ± SD)	Treatment diets[1]				
	Prepartum		Postpartum		
	Control	High	Control	High	Lactation[2]
DM, %[4]	46.4 ± 3.6	46.3 ± 3.3	42.6 ± 2.5	43.1 ± 2.4	42.5 ± 1.4
CP, % of DM	11.5 ± 0.4	14.6 ± 1.0	14.5 ± 0.3	15.9 ± 0.5	15.4 ± 0.6
aNDF, % of DM	43.4 ± 1.0	41.3 ± 0.4	33.3 ± 0.5	32.0 ± 0.5	27.6 ± 0.8
ADF, % of DM	28.2 ± 0.7	26.0 ± 0.3	21.7 ± 0.4	20.2 ± 0.5	17.4 ± 0.7
Lignin, % of DM	3.4 ± 0.2	3.0 ± 0.3	2.6 ± 0.2	2.2 ± 0.2	1.8 ± 0.2
Sugar, % of DM	3.8 ± 0.3	4.6 ± 0.3	4.8 ± 0.3	5.2 ± 0.5	5.0 ± 0.3
Starch, % of DM	21.8 ± 0.7	20.9 ± 0.9	24.7 ± 0.7	24.7 ± 0.9	32.1 ± 1.3
Fat, % of DM	3.4 ± 0.1	2.9 ± 0.1	4.9 ± 0.1	4.8 ± 0.1	4.2 ± 0.2
Ash, % of DM	7.7 ± 0.2	7.7 ± 0.2	8.2 ± 0.2	8.0 ± 0.2	6.8 ± 0.2
Ca, % of DM	1.52 ± 0.06	1.59 ± 0.15	1.14 ± 0.07	1.02 ± 0.13	0.93 ± 0.12
P, % of DM	0.43 ± 0.01	0.40 ± 0.02	0.39 ± 0.03	0.36 ± 0.01	0.44 ± 0.02
Mg, % of DM	0.45 ± 0.01	0.51 ± 0.03	0.43 ± 0.02	0.41 ± 0.01	0.32 ± 0.02
K, % of DM	1.03 ± 0.03	1.14 ± 0.05	1.49 ± 0.12	1.47 ± 0.05	1.47 ± 0.05
S, % of DM	0.48 ± 0.02	0.42 ± 0.02	0.23 ± 0.01	0.22 ± 0.02	0.22 ± 0.02
Na, % of DM	0.12 ± 0.02	0.11 ± 0.02	0.58 ± 0.04	0.53 ± 0.02	0.62 ± 0.11
Cl, % of DM	0.49 ± 0.03	0.58 ± 0.04	0.56 ± 0.02	0.51 ± 0.03	0.34 ± 0.03
DCAD, mEq/100 g	-12.1 ± 2.7	-8.7 ± 2.3	33.6 ± 5.1	32.6 ± 2.8	40.7 ± 6.3
ME Mcal/kg DM[5]	2.09	2.13	2.65	2.66	2.69
MP, g/kg DM[5]	87	115	101	127	113
RDP, % of DM[5]	7.46	9.04	9.06	8.92	10.46
RUP, % of DM[5]	3.71	7.08	5.00	8.25	6.39
Methionine, g/Mcal ME	1.24	1.23	1.18	1.20	1.01
Lysine, g/Mcal ME	3.88	3.84	3.27	3.31	2.88

[1]Prepartum TMRs, fed starting at 28 d before expected calving, were formulated to contain methionine and lysine at 1.24 and 3.84 g/Mcal metabolizable energy (ME), respectively and supply a control (C; 85 g MP/kg DM) or high (H; 113 g MP/kg DM) level of estimated metabolizable protein (MP). Postpartum TMRs, fed from 0 to 21 DIM, were formulated to contain methionine and lysine at 1.15 and 3.16 g/Mcal ME, respectively and supply control (C; 104 g MP/kg DM) or high (H; 131 g MP/kg DM) level of estimated MP.
[2]From 22 to 42 DIM, cows were fed a lactation diet that contained methionine and lysine at 1.01 and 2.88 g/Mcal ME, respectively and supplied 113 g of estimated MP/kg DM.

[3]Chemical composition represents the mean ± SD of 6 4-wk composite samples.

[4]Presented as mean ± SD of 22 weekly DM measurements.

[5]RDP = rumen degradable protein, RUP = rumen undegradable protein. Estimated in AMTS.Cattle.Professional v. 4.17.0.0 (AMTS LLC) using average dry matter intake for each diet.

Table 3. Prepartum dry matter intake, body weight and metabolizable energy and protein balance as well as colostrum production.

Variable	Treatment[1]		*P*-value		
	Control (n = 48)	High (n = 47)	Trt	Wk	Trt x Wk
DMI, kg/d	13.5 ± 0.3	14.0 ± 0.3	0.04	< 0.01	0.43
DMI, % BW	1.77 ± 0.03	1.80 ± 0.03	0.29	< 0.01	0.9
BW, kg	768 ± 3	778 ± 3	< 0.01	< 0.01	0.96
BW change, kg	23.9 ± 3.3	35.1 ± 3.3	< 0.01		
BW change, %	3.21 ± 0.45	4.78 ± 0.45	< 0.01		
BCS	3.39 ± 0.02	3.39 ± 0.02	0.76	0.40	0.35
BCS change, n (%)			0.14		
-0.25	4 (8.3)	2 (4.3)			
0.00	23 (47.9)	33 (70.2)			
0.25	20 (41.7)	12 (25.5)			
0.50	1 (2.1)	0 (0)			
MP supply, g/d[2]	1,180 ± 27	1,606 ± 27	< 0.01	< 0.01	0.06
MP balance, %[2]	101.1 ± 1.6	139.0 ± 1.6	< 0.01	< 0.01	< 0.01
ME balance, %[2]	96.7 ± 1.8	101.8 ± 1.8	< 0.01	< 0.01	0.79
Colostrum yield, kg	15.0 ± 1.0	15.9 ± 1.0	0.62		
Brix percentage, %	26.8 ± 0.5	27.0 ± 0.5	0.82		

[1]Prepartum TMRs, fed starting at 28 d before expected calving, were formulated to contain methionine and lysine at 1.24 and 3.84 g/Mcal metabolizable energy (ME), respectively and supply a control (C; 85 g MP/kg DM) or high (H; 113 g MP/kg DM) level of estimated metabolizable protein (MP).
[2]Estimated in AMTS.Cattle.Professional v. 4.17.0.0 (AMTS LLC) weekly using cow's DMI, BW, calf birth weight, and days carried calf.

Postpartum period

Results from the postpartum period are in Tables 4 and 5. Dry matter intake (% of BW) did not differ during the postpartum period ($P \geq 0.70$). Metabolizable protein supply from 1 to 21 DIM was greater for cows fed the postpartum H diet (CH: 2766 ± 50; HH: 2667 ± 51 g/d) compared to cows fed the C postpartum diet (CC: 2033 ± 51; HC: 2030 ± 52 g/d; $P < 0.01$). Milk yield during 1 to 21 DIM was greater in CH (42.4 ± 0.9 kg/d) compared to HC (38.0 ± 1.0 kg/d; $P < 0.01$) and milk yield in HH (44.7 ± 1.0 kg/d) was greater than CC (39.2 ± 1.0 kg/d; $P < 0.01$) and HC ($P < 0.01$), respectively. Concentrations of milk protein ($P = 0.15$), fat ($P = 0.79$), and total solids ($P = 0.90$) were not affected by treatment, but lactose was greater in CH compared to CC (4.81 ± 0.02 vs. 4.76 ± 0.02 %; $P = 0.04$). Milk urea nitrogen was lower in cows fed a control level of MP postpartum (CC: 6.37 ± 0.36; HC: 6.92 ± 0.36 mg/dL) compared to cows fed the H postpartum diet (CH: 8.83 ± 0.35; HH: 9.33 ± 0.35 mg/dL; $P < 0.01$). Cows fed CC had a greater loss in body weight (7.36 ± 0.65%) compared to cows fed CH (4.06 ± 0.64%; $P < 0.01$). Nevertheless, BCS ($P = 0.54$), the loss of BCS ($P = 0.42$), concentrations of circulating fatty acids ($P = 0.41$), and events of hyperketonemia ($P = 0.13$) did not differ by treatment during the first three weeks of lactation. Metabolizable protein supply was

greater in CH at wk 4 compared to CC (P = 0.03) and HC (P = 0.03), but MP supply did not differ by treatment at wk 5 or 6 (P > 0.12). Milk yield remained elevated from 22 to 42 DIM in the cows fed the high postpartum diet (CH: 53.3 ± 1.0; HH: 54.1 ± 1.0 kg/d) compared to the cows fed the control postpartum diet (CC: 49.6 ± 1.0; HC: 49.3 ± 1.0 kg/d). Concentrations of milk components did not differ by treatment (P ≥ 0.10). Although the change in body weight did not differ from 22 to 42 DIM, BCS was lower in CC compared to CH (3.04 ± 0.03 vs. 3.17 ± 0.03; P = 0.02). From 7 to 40 DIM, backfat thickness (P = 0.99) as well as longissimus dorsi muscle diameter (P = 0.78) did not differ by treatment. Further, treatment did not affect projected 305-d milk production at last test (M305; CC: 12,076 ± 419; CH: 12,865 ± 410; HC: 11,797 ± 429; HH: 12,886 ± 419 kg; P = 0.17).

Conclusions and Implications

Increasing prepartum MP supply from 1,180 to 1,606 g/d did not affect colostrum yield or Brix percent from multiparous cows. Moreover, when feeding a control level of MP postpartum (~2,030 g/d), these data do not support increasing prepartum MP supply >1,200 g/d based on the lack of difference in milk yield during early lactation (CC vs. HC). However, lactation performance was increased in response to feeding a high level of MP (~2,700 g/d) in the fresh diet and these differences in performance persisted beyond the initial feeding period. Notably, cows fed a high level of MP during the pre- and postpartum period (HH) produced the most milk during wk 1 which resulted in a numerically higher milk yield (+2.3 ± 1.0 kg/d; 5.1 ± 2.2 lb/d) during the first three weeks of lactation when compared to cows fed CH. Overall, these data support increasing the MP supply during the postpartum period and under the correct economic situation, producers might benefit from feeding a high level of MP pre- and postpartum.

Table 4. Dry matter intake, BW, milk production, and metabolizable energy and protein balance from 1 to 21 DIM.

	Treatment[1]				*P*-value		
Variable	CC (n = 23)	CH (n = 24)	HC (n = 22)	HH (n = 23)	Trt	Wk	Trt x Wk
DMI, kg/d	20.2 ± 0.4	21.5 ± 0.4	20.5 ± 0.4	20.7 ± 0.4	0.10	< 0.01	0.68
DMI, % BW	2.98 ± 0.06	3.04 ± 0.06	3.00 ± 0.06	2.94 ± 0.06	0.71	< 0.01	0.52
Milk Yield, kg/d	39.2 ± 1.0^{bc}	42.4 ± 0.9^{ab}	38.0 ± 1.0^{c}	44.7 ± 1.0^{a}	< 0.01	< 0.01	< 0.01
ECM, kg/d	46.4 ± 1.1^{bc}	50.3 ± 1.1^{ab}	45.2 ± 1.1^{c}	53.6 ± 1.1^{a}	< 0.01	< 0.01	< 0.01
Fat, %	4.81 ± 0.11	4.82 ± 0.11	4.84 ± 0.12	4.96 ± 0.11	0.79	< 0.01	0.26
Protein, %	3.20 ± 0.04	3.20 ± 0.04	3.25 ± 0.04	3.11 ± 0.04	0.15	< 0.01	0.16
Lactose, %	4.76 ± 0.02^{b}	4.81 ± 0.02^{a}	4.81 ± 0.02^{ab}	4.79 ± 0.02^{ab}	0.04	< 0.01	0.88
Total Solids, %	13.77 ± 0.14	13.84 ± 0.13	13.90 ± 0.14	13.87 ± 0.13	0.90	< 0.01	0.81
MUN, mg/dL	6.37 ± 0.36^{b}	8.83 ± 0.35^{a}	6.92 ± 0.36^{b}	9.33 ± 0.35^{a}	< 0.01	0.30	0.38
BW, kg	678 ± 11^{bc}	716 ± 11^{ab}	676 ± 11^{c}	720 ± 11^{a}	< 0.01	< 0.01	0.05
BW change, kg	-55 ± 5^{b}	-30 ± 5^{a}	-42 ± 5^{ab}	-46 ± 5^{ab}	< 0.01		
BW change, %	-7.36 ± 0.65^{b}	-4.06 ± 0.64^{a}	-5.63 ± 0.67^{ab}	-6.12 ± 0.67^{ab}	< 0.01		
BCS	3.27 ± 0.04	3.33 ± 0.03	3.31 ± 0.04	3.33 ± 0.04	0.54	< 0.01	0.18
BCS change, n (%)					0.42		
-0.75	0 (0)	0 (0)	0 (0)	1 (4.4)			
-0.50	6 (26.1)	2 (8.4)	2 (9.1)	3 (13.0)			
-0.25	11 (47.8)	11 (45.8)	13 (59.1)	13 (56.5)			
0.00	6 (26.1)	11 (45.8)	7 (31.8)	5 (21.8)			
0.25	0 (0)	0 (0)	0 (0)	1 (4.3)			
MP supply, g/d[2]	2,033 ± 51^{b}	2,766 ± 50^{a}	2,030 ± 52^{b}	2,667 ± 51^{a}	< 0.01	< 0.01	0.20
MP balance, %[2]	70.7 ± 1.3^{b}	92.1 ± 1.3^{a}	71.9 ± 1.3^{b}	87.4 ± 1.3^{a}	< 0.01	< 0.01	0.30
ME balance, %[2]	75.3 ± 1.6^{ab}	77.4 ± 1.6^{a}	76.8 ± 1.6^{ab}	71.6 ± 1.6^{b}	0.05	< 0.01	0.56

[a-c] Least squares means with different superscripts differ ($P \leq 0.05$; Tukey's test).

[1]Prepartum TMRs, fed starting at 28 d before expected calving, were formulated to contain methionine and lysine at 1.24 and 3.84 g/Mcal metabolizable energy (ME), respectively and supply a control (C; 85 g MP/kg DM) or high (H; 113 g MP/kg DM) level of estimated metabolizable protein (MP). Postpartum TMRs, fed from 0 to 21 DIM, were formulated to contain methionine and lysine at 1.15 and 3.16 g/Mcal ME, respectively and supply control (C; 104 g MP/kg DM) or high (H; 131 g MP/kg DM) level of estimated MP. The combination of a pre- and postpartum diet resulted in treatments: 1) CC, 2) CH, 3) HC, 4) HH. From 22 to 42 DIM, cows were fed a lactation diet that contained methionine and lysine at 1.01 and 2.88 g/Mcal ME, respectively and supplied 113 g MP/kg.

[2]Estimated in AMTS.Cattle.Professional v. 4.17.0.0 (AMTS LLC) weekly using cow's DMI, BW, DIM, milk yield, and milk composition.

Table 5. Dry matter intake, BW, milk production, and metabolizable energy and protein balance from 22 to 42 DIM.

	Treatment[1]				*P*-value		
Variable	CC (n = 23)	CH (n = 24)	HC (n = 22)	HH (n = 23)	Trt	Wk	Trt x Wk
DMI, kg/d	25.1 ± 0.5	26.8 ± 0.5	25.1 ± 0.5	25.6 ± 0.5	0.06	< 0.01	< 0.01
DMI % BW	3.86 ± 0.08	3.89 ± 0.07	3.82 ± 0.08	3.77 ± 0.08	0.70	< 0.01	< 0.01
Milk Yield, kg/d	49.6 ± 1.0^{b}	53.3 ± 1.0^{a}	49.3 ± 1.0^{b}	54.1 ± 1.0^{a}	< 0.01	< 0.01	0.21
ECM, kg/d	52.8 ± 1.1^{b}	57.9 ± 1.1^{a}	52.8 ± 1.1^{b}	57.7 ± 1.1^{a}	< 0.01	< 0.01	0.53
Fat, %	4.09 ± 0.09	4.28 ± 0.09	4.10 ± 0.10	4.11 ± 0.09	0.38	< 0.01	0.62
Protein, %	2.83 ± 0.03	2.82 ± 0.03	2.91 ± 0.03	2.79 ± 0.03	0.10	0.02	0.08
Lactose, %	4.85 ± 0.01	4.86 ± 0.01	4.88 ± 0.01	4.85 ± 0.01	0.42	< 0.01	0.69
Total Solids, %	12.69 ± 0.11	12.89 ± 0.11	12.84 ± 0.11	12.70 ± 0.11	0.45	< 0.01	0.41
MUN, mg/dL	8.96 ± 0.31	9.61 ± 0.31	9.46 ± 0.32	9.86 ± 0.32	0.15	< 0.01	0.07
BW, kg	650 ± 10^{b}	689 ± 10^{a}	657 ± 10^{ab}	681 ± 10^{ab}	0.02	0.16	0.92
BW change, kg	-0.5 ± 4.3	4.1 ± 4.2	-0.1 ± 4.4	2.2 ± 4.3	0.82		
BW change, %	-0.1 ± 0.7	0.7 ± 0.6	0.1 ± 0.7	0.4 ± 0.7	0.83		
BCS	3.04 ± 0.03^{b}	3.17 ± 0.03^{a}	3.08 ± 0.04^{ab}	3.10 ± 0.03^{ab}	0.03	< 0.01	0.74
BCS change					0.98		
-0.50	1 (4.4)	2 (8.3)	1 (4.5)	2 (8.7)			
-0.25	6 (26.1)	7 (29.2)	8 (36.4)	5 (21.7)			
0.00	13 (56.5)	13 (54.2)	10 (45.5)	14 (60.9)			
0.25	3 (13.0)	2 (8.3)	3 (13.6)	2 (8.7)			
MP supply, g/d[2]	2,801 ± 67^{b}	3,079 ± 65^{a}	2,798 ± 68^{b}	2,926 ± 67^{ab}	< 0.01	< 0.01	0.01
MP balance, %[2]	94.3 ± 1.5	98.3 ± 1.4	93.3 ± 1.5	94.1 ± 1.5	0.07	< 0.01	< 0.01
ME balance, %[2]	88.5 ± 1.7	88.2 ± 1.7	88.7 ± 1.8	85.0 ± 1.7	0.39	< 0.01	0.60

[a-c] Least squares means with different superscripts differ ($P \leq 0.05$; Tukey's test).

[1]Prepartum TMRs, fed starting at 28 d before expected calving, were formulated to contain methionine and lysine at 1.24 and 3.84 g/Mcal metabolizable energy (ME), respectively and supply a control (C; 85 g MP/kg DM) or high (H; 113 g MP/kg DM) level of estimated metabolizable protein (MP). Postpartum TMRs, fed from 0 to 21 DIM, were formulated to contain methionine and lysine at 1.15 and 3.16 g/Mcal ME, respectively and supply control (C; 104 g MP/kg DM) or high (H; 131 g MP/kg DM) level of estimated MP. The combination of a pre- and postpartum diet resulted in treatments: 1) CC, 2) CH, 3) HC, 4) HH. From 22 to 42 DIM, cows were fed a lactation diet that contained methionine and lysine at 1.01 and 2.88 g/Mcal ME, respectively and supplied 113 g MP/kg.

[2]Estimated in AMTS.Cattle.Professional v. 4.17.0.0 (AMTS LLC) weekly using cow's DMI, BW, DIM, milk yield, and milk composition.

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Feeding Management in the AMS: Limits to Precision Feeding Approaches

G. Penner[1] and T. DeVries[2]
[1]Department of Animal & Poultry Science, University of Saskatchewan, Saskatoon, SK
[2]Department of Animal Biosciences, University of Guelph, Guelph, ON'

Introduction

There are two main goals of the nutritional program for cows milked with automated milking systems (AMS). The first, is to stimulate cows to voluntarily enter the AMS by providing a nutritional reward in the AMS. It is clear that removal of the nutritional reward compromises voluntary attendance to the AMS or pre-selection area (Jago et al., 2007; Scott et al., 2014; Shortall et al., 2018) along with milk yield. Moreover, altering the composition of the feed provided in the AMS can further enhance motivation to enter (Madsen et al., 2010; Johnson et al., 2022). The second goal, as with all planned nutritional programs, is to provide a diet that meets nutrient requirements for maintenance and production. However, with AMS systems, dietary components are provided in a partial mixed ration at a common feed bunk and within the AMS. There is a perception that altering the quantity or type of concentrate provided in the AMS allow dietary specification at a cow level. However, there are very few studies testing the ability to use precision feeding approaches.

In one study (Maltz et al., 2013) testing the concept of precision feeding, energy balance was determined weekly by measuring cow BW, milk energy output and DMI. The diet was then reformulated on a weekly basis for each cow to enable delivery of energy exceeding requirements by 5 Mcal/d. When nutrient consumption and nutrient utilization are known, applying the precision feeding approach increased milk yield and milk energy output, avoided extremes in energy balance, and limited changes in BW. While only 1 study and clearly challenging from a practical point of view, it is an excellent example for the application of precision feeding and opportunities that can arise.

Can We Apply Precision Feeding Strategies in AMS Systems?

For precision feeding systems to be effective in AMS systems, concentrate intake in the AMS and consumption of the PMR must be known. Moreover, varying the amount of concentrate must lead to predictable changes in AMS concentrate and PMR intake. The data are clear that increasing the quantity of AMS pellet offered in the AMS increases the day-to-day variability in the delivery of of the AMS pellet (Bach et al., 2007; Bach and Cabrera, 2017) and this response occurs in both guided (Hare et al. 2018; Menajovsky et al. 2018; Paddick et al. 2019) and free-flow traffic systems (Henriksen et al. 2019; Schwanke et al. 2019). Based on the available data from our laboratory (Hare et al. 2018; Menajovsky et al. 2018; Paddick et al. 2019), the coefficient of variation (CV) in AMS pellet delivered averages 13.5%. Using this CV, we can calculate the standard deviation for AMS pellet delivery by multiplying the amount delivered by the CV (Figure 1). A more

recent study has reported a CV value of 13.0% (Schwanke et al., 2022). Using this approach, it is clear that as the amount of AMS pellet delivered increases, the day-to-day variation in the amount delivered also increases. In fact, we would expect that the day-to-day variation in the amount of pellet delivered for 96% of the cows would increase from 0.54 kg/day to 2.7 kg/day as the AMS pellet delivered increases from 2 to 10 kg/day. Using a 10 kg/day value and a fixed DMI of 28 kg/day, we would expect that AMS pellet would range between 8.7 and 11.4 kg/day. If we assume that total DMI (AMS pellet + PMR) is relatively constant, the variability in AMS pellet delivery could imply that PMR intake could also vary from 19.4 to 16.7 kg/d. However, the amount of pellet offered in the AMS did not affect PMR intake or variability in PMR intake in previous studies in guided (Hare et al. 2018; Menajovsky et al. 2018; Paddick et al. 2019) or free-flow barns (Henriksen et al. 2019; Schwanke et al. 2019, 2022). Similar to increased day-to-day variation for AMS concentrate offered within a cow, one study has reported that with increasing AMS allocation there is greater variation among cows that should receive the same AMS allocation (Henriksen et al., 2019). The greater variability among cows may create additional challenges as nutritionists work to troubleshoot and improve farm performance indicators.

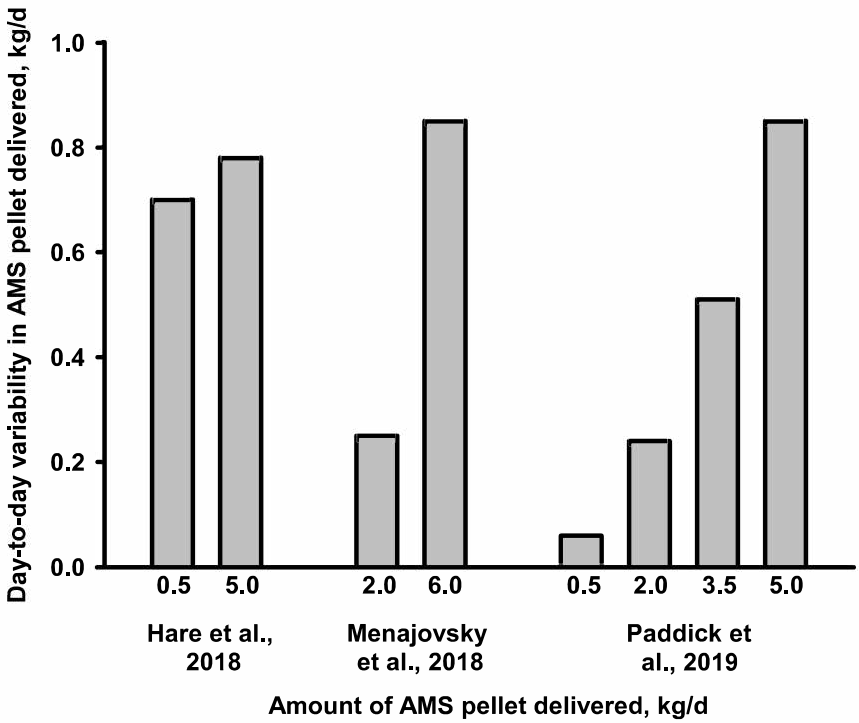

Figure 1. Variability in day-to-day pellet delivered in the AMS based on the amount of pellet offered in the AMS.

In addition to variation in the amount of concentrate delivered on a daily basis, cows offered more concentrate in the AMS also leave more concentrate behind as they exit the AMS (Bach and Cabrera, 2017). Unfortunately, very few AMS have the ability to remove or record the amount of concentrate left as refusals in the manger. In one such study, it was shown that increasing the amount of pellet offered in the AMS resulted in

greater quantities of pellet refusals and that refusals were greater for Holstein than Jersey and greater for primiparous than multiparous cows (Henriksen et al. 2019).

Providing more concentrate in the AMS does not necessarily translate to greater DMI (Table 1). For example, Hare et al. (2018) reported that for every 1 kg increase in AMS pellet delivered, there was a corresponding decrease in PMR DMI of 1.58 kg.Bach et al. (2007) reported a 1.14 kg reduction in PMR DMI and Paddick et al. (2019) reported that PMR DMI decreased by 0.97 kg for every one kg DM increase in AMS pellet delivered while. More recently, a substitution of up to 5:1 has been reported for cows in early lactation (Henriksen et al., 2019). A study targeting substation rate has demonstrated that PMR characteristics influence the response (Menajovsky et al., 2018) providing some of the first known data evaluating reasons for substitution. The variable reduction in PMR DMI with increasing AMS concentrate intake may imply that nutrient intake may not be positively affected. In contrast, Schwanke et al. (2019) reported that for every 1 kg increase in AMS pellet intake there was only a 0.63 kg reduction in PMR DMI. In that case, providing more pellet in the AMS resulted in greater total DMI and likely explains the numerical improvement in milk yield observed in that study. The variable and currently unpredictable substitution rate may challenge the ability to formulate diets for individual cows in the same pen given that only the amount or types of concentrate in the AMS can differ. It should be noted that the inability to predict the substitution rate (and hence PMR intake) does not preclude imposing such precision feeding programs; we simply cannot evaluate the individual response or adequately predict the outcome. Clearly, this remains a challenge for nutritionists and producers alike.

Automated milking systems also enable producers to impose adaptation programs for cows in early lactation. While increasing the energy density of the diet by increasing pellet allocation may seem like a plausible option, recent results suggest that such an approach may actually decrease DMI and milk yield (Deiho et al., 2016). Few studies have been conducted to test these responses for cows in AMS. In an unpublished study (Haisan et al. unpublished) we tested providing 3 vs. 8 kg of pellet in the AMS with cows provided 8 kg divided into a rapid adaptation (concentrate increased from 3 kg to 8 kg in 5 d) or moderate rate of adaptation group (3 kg to 8 kg in 14 d). Rate of adaptation did not affect responses, but cows offered the high pellet allocation never consumed their target AMS pellet, had lower PMR intake, tended to visit the AMS less frequently, and had lower milk fat yield. In addition, Henriksen et al. (2019) reported that early lactation cows did not produce more milk or energy corrected milk when offered a greater quantity of concentrate in the AMS. Clearly there is a need for future research under AMS conditions to help understand factors that influence ability to deliver a specific diet to individual cows.

Table 1. Effect of concentrate allocation in the AMS on substitution of the concentrate for the PMR. The substitution rate indicates the quantity of PMR intake reduction (DM basis) for every 1 kg increase in AMS concentrate consumed. Studies highlighted in grey are from free flow traffic systems and studies without shading are from guided flow systems.

Study	DIM (Average ± SD)	Cows, parity, and study design	Dietary Strategy	Substitution Ratio (kg DM)
Bach et al., 2007	191 ± 2.13	69 Primiparous and 46 Multiparous, Completely randomized	Isocaloric	1.14
Hare et al., 2018	227 ± 25 123 ± 71	5 Multiparous and 3 Primiparous, Cross-over	Isocaloric	1.58
Henriksen et al., 2018	32-320 14-330	22 primiparous Holstein 19 multiparous Holstein 11 wk study	Static PMR with 2 concentrate	0.58 – 0.92
Henriksen et al., 2018	29-218 17-267	14 primiparous Jersey 28 multiparous Jersey 11 wk study	Static PMR with 2 concentrate	0.69-0.50
Menajovsky et al., 2018	141 ± 13.6	8 Multiparous, Replicated 4 ´ 4 Latin square	LF-PMR HF-PMR	0.89 0.78
Henriksen et al., 2019	Early (5 to 14) Mid (15 to 240) Late (240 to 305)	Continuous lactation study 128 cows (68 Holstein + 60 Jersey)	Static PMR	5 1.1 2.9
Paddick et al., 2019	90.6 ± 9.8	8 Primiparous, Replicated 4 ´ 4 Latin square	Isocaloric	0.97
Schwanke et al., 2019	47.1 ± 15.0	15 Primiparous, Cross-over	Isocaloric	0.62

Does Increasing the AMS Concentrate Allocation Increase Voluntary Attendance and Milk Yield?

One of the most common claims with AMS feeding strategies is that increasing the amount of pellet delivered in the AMS will stimulate voluntary attendance and milk yield. While there are studies partially or fully supporting this claim (Scott et al., 2014; Schwanke et al., 2019), there are also numerous that contradict that claim (Table 2). Variation responses have been attributed to wide range of possible explanations including traffic flow, stage of lactation for the cows in the study, forage quality in the PMR, diet formulation strategy, and composition of the pellet. In addition, it is likely that magnitude of substitution of the AMS concentrate and PMR influence whether voluntary visits and milk yield are affected.

Table 2. Summary of studies evaluating AMS feeding strategies and their response for voluntary visits and milk yield. Studies highlighted in grey are from free flow traffic systems and studies without shading are from guided flow systems.

Study	DIM (Average ± SD)	Cows, parity, and study design	Dietary Strategy	Visit or milk yield response
Halachmi et al., 2005	Not described	453 cows Parity not described	Common PMR 2 amounts of concentrate	Increased yield, no change in visits
Bach et al., 2007	191 ± 2.13	69 Primiparous and 46 Multiparous, Completely randomized	Isocaloric PMR with 3 vs. 8 kg of pellet	No
Tremblay et al., 2016	Not described	Herd-based analysis	Herd-based comparison	Decreased
Henriksen et al., 2018	32-320 14-330	22/14 primiparous Holstein/Jersey 19/28 multiparous Holstein/Jersy 11 wk study	Common PMR with 2 amounts of concentrate	Increased yield, no change in visits
Henriksen et al., 2019	Early (5 to 14) Mid (15 to 240) Late (240 to 305)	Continuous lactation study 128 cows (68 Holstein + 60 Jersey)	Common PMR with 2 amounts of concentrate	No
Schwanke et al., 2019	47.1 ± 15.0	15 Primiparous, Cross-over	Isocaloric PMR with 2 vs. 6 kg pellet	Increased visits, numeric yield
Schwanke et al., 2022	123.9 ± 53.2 DIM	15 multiparous, Cross-over	Common PMR with 2 vs. 6 kg pellet	No
Hare et al., 2018	227 ± 25 123 ± 71	5 Multiparous and 3 Primiparous, Cross-over	Isocaloric with 2 amounts of concentrate	No
Menajovsky et al., 2018	141 ± 13.6	8 Multiparous, Replicated 4 ´ 4 Latin square	2 PMR energy densities and 2 amounts of concentrate	Tendency for visits and yield
Paddick et al., 2019	90.6 ± 9.8	8 Primiparous, Replicated 4 ´ 4 Latin square	Isocaloric with 4 amounts of concentrate	No
Haisen et al., unpublished	0 to 56	20 Holstein cows/treatment Low (3 kg/d), rapidly adapted to 8 kg/d, or gradually adapted to 8 kg/d	Common PMR with 3 vs. 8 kg/d	Less visits, reduced milk fat yield

Is the AMS pellet likely to induce ruminal acidosis?

There is often concern about risk for ruminal acidosis with AMS because a component feeding system is imposed and large quantities of pelleted feed may be programmed to be offered through the AMS. We have recently reported that the PMR formulation, rather than the quantity of pellet in the AMS, has a greater impact on ruminal pH (Menajovsky et al., 2018). It is logical that the PMR had greater impact than the AMS pellet considering it accounted for over 80% of the DMI in that study. Additionally, AMS pellet meal size in that study was constrained to a maximum of 2.5 kg and the amount delivered in the AMS was managed to not exceed 6 kg/cow/day on a DM basis. Based on recent information, cows in commercial operations may be provided up to 11.2 kg (as fed basis) of pellet in the AMS (Salfer and Endres, 2018). With this strategy, large swings in dietary composition can occur based on the expected reduction in PMR intake and increased pellet intake in the AMS. Under such scenarios, we could expect that the dietary physically effective NDF content would be dramatically reduced (and potentially deficient) and that ruminally degradable carbohydrate content would increase thereby creating a diet (PMR + AMS pellet) that could be perceived to be high risk for ruminal acidosis. Currently, there are no data to support or dispute the previous claim.

How important is the type of supplement provided in the AMS?

In addition to general feeding management, palatability of the pellet provided in the AMS is also important. Madsen et al. (2010) evaluated pellets containing barley, wheat, a barley-oat mix, maize, artificially dried grass, or pellets with added lipid with all cows fed a common PMR. They observed that AMS pellet intake and voluntary visits were greatest when the pellets contained the wheat or the barley-oat mix. However, pelleted barley and wheat are expected to have a rapid rate of fermentation in the rumen and feeding substantial quantities would be expected to increase the risk for low ruminal pH. To reduce fermentability, pellets could be prepared with low-starch alternatives (Miron et al., 2004; Halamachi et al., 2006 and 2009). Substituting starch sources with soyhulls did not negatively affect voluntary attendance at the AMS or milk yield (Halamachi et al., 2006, 2009), and may slightly improve milk fat and reduce milk protein concentrations (Miron et al., 2004).

Producers may also choose to use home-grown feeds in the AMS. In a recent study, we tested whether feeding a pellet was required or if we could deliver steam-flaked barley as an alternative (Johnson et al., 2022) in a feed-first guided-traffic flow barn. In that study, the pellet comprised only barley grain and the same source of barley grain was used for the steam-flaked treatment. In all cases, cows were programmed to have 2.0 kg of the concentrate in the AMS delivered. While PMR (27.0 kg/d DM basis) and AMS concentrate intake (1.99 kg/d DM basis) did not differ among treatments, cows fed the steam-flaked barley tended to have fewer visits (2.99 vs. 2.83) to the AMS, tended to have a longer interval between milking events (488 vs. 542 min), and spent 28 minutes more in the holding area prior to entering the AMS than those fed pelleted barley. While this did not translate into differences in milk yield (average of 44.9 L/d), it may be expected that with a longer-term study, production impacts would be observed. In contrast, Henriksen et al.

(2018) reported greater voluntary visits when a texturized feed (combination of pellet and steam-rolled barley) was provided in comparison to a pellet alone. Regardless, utilization of a pellet as the sole ingredient or part of the mix may limit the ability of producers to use home-grown feeds in the AMS.

Partial Mixed Ration: The Major, but Forgotten Component of the Diet

As mentioned previously, all surveys that have been published to date focus on AMS feeding with little or no information collected to describe PMR composition or intake. The lack of focus on the PMR is likely because only group intakes can be determined and many of the studies have been conducted using retrospective analysis. However, drawing conclusions or making recommendations for feeding management without considering the PMR could lead to erroneous decisions. We recently completed a study where we varied the formulation of the PMR such that we increased the energy density of the PMR by a similar magnitude to that commonly used when increasing the amount of pellet in the AMS (Menajovsky et al., 2018). Feeding the PMR with a greater energy density tended to increase milk yield (39.2 vs. 37.9 kg/d; P = 0.10) likely because of greater energy supply. In several studies we have also noted that formulation of the PMR impacts sorting characteristics of the PMR (Menajovsky et al., 2018;Paddick et al. 2019). In both cases, reducing the energy density of the PMR (greater forage content as a percentage of DM) increased the sorting potential of the PMR. This may lead to cows selecting for dietary components in an undesirable manner (Miller-Cushon and DeVries, 2017).

More recently, survey-based studies have confirmed that factors such as greater bunk space and more frequent PMR push-ups improve milk yield responses for cows in AMS(Matson et al., 2021). These findings supported previous research by Siewert et al. (2018) highlighting that management factors associated with the PMR are important factors that can affect the success of AMS. Future research is needed to understand how PMR feeding management and PMR composition affect the ability to stimulate voluntary visits and to meet nutrient requirements for cows milked with AMS.

Conclusions

While a commonly stated goal of AMS is to enable precision feeding strategies, current data have highlighted a few key challenges that must be addressed. Specifically, ensuring cows are delivered and eat the AMS allocation is one hurdle along with the ability to predict or measure PMR intake and the change in intake that occurs with increasing AMS concentrate allocation. As such, precision feeding cannot solely focus on AMS concentrate feeding, but rather must consider whole farm management and specifically management of the PMR.

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What We've Learned from Cows:
A Tale of Two Decades of Management Research at Miner

R. J. Grant
William H. Miner Agricultural Research Institute, Chazy, NY

Introduction

Earlier this year, Larry Chase asked me to prepare a paper called "*What I've Learned from Cows*" that would summarize three decades of dairy cattle research. I have switched the "I" to "we" and will present my perspectives on management research conducted by our Miner Institute team in the past 20 years and what the cows have taught us!

The research began with understanding behavioral time budgets and defining the concept of "cow comfort economics." An essential insight was that we *must accommodate the dairy cow's natural behaviors* – eating, resting, and ruminating – within an optimized management environment to enhance productivity, well-being, and herd profitability. Nutrition models improve rapidly as new biology is incorporated; attendees at the Cornell Nutrition Conference hear the latest advancements in the Cornell Net Carbohydrate and Protein System (CNCPS) every year. But the cow tells us that poor management impedes her ability to respond to the diet. Work by Bach et al. (2008) quantitated the overriding impact of non-dietary factors, such as feed availability and stall stocking density, on farm-to-farm variability in milk production relative to nutritional factors. Consequently, a long-term goal of Miner research has been to incorporate cow management into nutrition models.

An accurate statement of what we've learned from cows can be summarized as: *cows that aren't rushed while eating, have the freedom to lie down and ruminate, and can strike the correct balance between eating and recumbent rumination, will have optimal rumen conditions for fiber digestion, microbial growth, and healthy production of more milk components.* Explaining how I arrived at this statement is the objective of this paper.

Time Budgets and Cow Comfort Economics

We routinely monitor time budget behaviors to assess the quality of herd management. But what is commonplace today was not in the 1970s and earlier, and we can thank Jack Albright of Purdue University for his pioneering insight into the value of studying cow behavior to improve performance and well-being. In the June 2023 issue of the Miner Institute Farm Report (Grant, 2023), I explained how Albright and his team in 1975 recorded the daily behavior of the first cow to break 22,676 kg of milk/yr (i.e., 50,000 lb/yr), Beecher Arlinda Ellen. This record-setting cow spent approximately 6 h/d eating and nearly 14 h/d lying down. Her daily dry matter intake (DMI) approached an enormous 7% of bodyweight. She spent 7.5 h/d ruminating with about 93% of that rumination occurring while lying down (i.e., while sternally recumbent). Albright suggested that

sternal recumbency, with a slight left-side laterality, allowed the cow to remain comfortable and for the rumen to function optimally. Given Ellen's prodigious productivity it is unsurprising that recently published research has reported a positive relationship between recumbent rumination and feed intake, milk fat, and milk protein percentage (McWilliams et al., 2022). More on this relationship later.

Conversations with Albright in the mid- to late 1990s led to an active interest in assessing cow behavior on commercial dairy farms. Baseline behavioral data collected by Bill Matzke (2003) as part of his MS research at the University of Nebraska on commercial dairy farms helped develop realistic and useful time budgets for lactating cows. Insights from this early work on time budgeting were: 1) excessive time outside the pen has a profound negative impact on resting behavior; 2) cows locked in headlocks beyond 1 h stop eating, and although 30 to 40% begin to ruminate while standing, they are essentially wasting their time; 3) the highest milk-producing cows in a pen spend up to 14 h/d resting (as did Beecher Arlinda Ellen); and 4) cows forced by poor management to spend more time on non-eating activities borrow that time from another one – most often resting. This early work highlighted the natural relationship between eating and resting, and how this interplay profoundly affects production and health.

Research at Miner Institute in the early 2000s cemented the idea that eating, resting, and ruminating are linked biologically – forming the foundation of cow comfort and what we called cow comfort economics (Grant, 2003; 2004; 2015). In many ways, we rediscovered previously reported information on the connection between eating and resting, and the priority that cows place on resting over eating behavior. Time outside the pen was crucial, given its impact on time available for eating, resting and other behaviors, and was viewed as the most vital component of dairy cow well-being in a competitive free-stall environment. Time outside the pen literally sets the limit on what is possible behaviorally within the pen. An Excel spreadsheet (Time Budget Evaluator, version 3. www.whminer.org; described by Grant, 2004) was developed to assess the time budget behaviors of cows and the potential for lost production in competitive environments.

Stocking Density and Overcrowding from the Cow's Perspective

Twenty years ago, it was already clear that overcrowding was becoming a significant management challenge in the dairy industry. When a pen of cows is overstocked, availability of resting and feeding space, area per cow, and access to water are all restricted. Studies conducted in the past 20 years have amply demonstrated that overcrowding, especially beyond 120% of stalls, hampers lying time, boosts lameness, reduces milk yield, encourages undesirable feeding behaviors (such as slug feeding and sorting), and elevates somatic cells (reviewed by Grant 2007; 2015). Early on, we compared alternative models for studying stocking density and concluded that simply denying resting and feeding space to simulate overcrowded 4-row housing was bioequivalent to more complicated research models (Krawczel et al, 2012b).

Defining the differential effect of overcrowding in a 4- or 6-row barn has not been rigorously examined with controlled research, yet we can safely infer that any specific stall stocking density will have potentially greater negative effects in a 6- versus a 4-row barn. Importantly, we have proposed that *time outside the pen interacts with stocking density*. Even at 100% stall stocking in a 4-row barn, time outside the pen of 6 versus 3 h/d reduced resting time by 2.6 h/d for multiparous cows and 4.2 h/d for primiparous cows in commingled pens (Matzke, 2003). It is difficult to name many factors that would affect resting so substantially.

Parity and Lameness Affect Response to Overcrowding

Soon after my arrival at Miner Institute, Chris Hill began a series of studies aimed at defining the short-term effects of overcrowding on behavior, performance, and how the response differed by parity and lameness. Hill et al. (2006; 2009) homed in on how stocking density affected primi- versus multiparous cows. As stall and headlock stocking density in 2-row pens increased from 100 to 142%, the difference in milk yield between multi- and primiparous cows increased from 2.8 kg/d at 100% up to 9.6 kg/d for 130% stocking density. At 142% stocking density, the difference dropped to 6.8 kg/d reflecting a negative effect on the dominant, multiparous cows as well as the subordinate, primiparous cows at this elevated level. Similarly, the difference in milk yield between sound and lame cows increased by 11.9 kg/d as stocking density of stalls and headlocks increased. Reinforcing the importance of resting rumination, these reductions in milk yield tracked with losses in both lying and ruminating, particularly for the lame cows.

What Stocking Density Does the Cow Actually Experience?

In this early work, we observed that sometimes daily rumination time was affected by stocking density, but not always (Hill, 2006; Krawczel et al., 2012a). But in all our studies rumination while lying down decreased at higher stocking densities. Furthermore, one study noted that subordinate cows lying in stalls preferred by dominant cows spent 40% less time ruminating while lying in the stall (Krawczel, 2007, unpublished). Given the recently recognized importance of recumbent rumination on DMI and milk components, this reduction in resting rumination with higher stocking density looms large as an economically important management challenge. An overarching management question must be: *what stocking density does an individual cow experience relative to the measured value (i.e., cows/stall)?* Beyond the number of cows per stall, it seems likely that subordinate cows faced with using stalls preferred by dominant cows must experience a functionally higher stocking density than the calculated one.

Currently we are evaluating the importance of location preference within a pen, whether free stalls, alley, or feed bunk. If individual cows, or types of cows, prefer to eat or rest only in certain areas of a pen then that would also affect their perceived level of competition for the resource and the optimal management practice to ensure adequate access for all cows in the pen. For example, Hefter et al. (2023) reported that lame cows preferred to use the free stalls nearest the pen exit. Cows, regardless of lameness status, also exhibited a strong preference for eating at feed bunk sections closest to the exit gate

between ~6:00 am and 9:00 pm, but not at night, presumably in relation to milking and feed availability. We plan future research on this topic.

De Vries et al. (2016) published a model that predicts changes in performance and profit per stall with variable stall stocking densities. A central relationship in this model of a loss of 0.50 kg/d of milk per 10% greater stall stocking density is based on Grant (2011), Fregonesi et al. (2007), and Bach et al. (2008). This useful model is available on the University of Florida web site: https://edis.ifas.ufl.edu/publication/AN346.

Assessing Cow Comfort in Overcrowded Pens

Increasing stocking density beyond 100 to 113% with our research model clearly increases the proportion of cows standing in alleys and compromises their ability to access free stalls when motivation to lie down is greatest. Pete Krawczel led research demonstrating that assessing cow comfort on a pen level is best done using the stall use index which is calculated as the number of cows in a pen lying down divided by the total number of cows minus those actively eating (Krawczel et al., 2008). In other words, the denominator includes cows that are essentially wasting their time, idling and mainly waiting to use a stall. The commonly used cow comfort index and stall standing index, calculated using only the cows lying or standing in a stall, remain relatively unchanged at higher stocking densities. For indices routinely measured and monitored on-farm, the stall use index and the rumination index (% of cows ruminating that are lying in stalls) top our list of priority indices. Campbell (2017) found that the commonly used rumination index is related to 24-h rumination time. Plus, the rumination index allows us to monitor recumbent rumination specifically.

Feed and Feeding Environment: Focus on Rumen pH

Overcrowding as a Subclinical Stressor

Given the wide array of negative behavioral, health, and performance consequences of overcrowding measured in research studies, the diversity of herd responses to overcrowding seems puzzling. Some herds seem to be immune to the negative consequences of overcrowding whereas others experience severe effects at low stall stocking density, and everything in between. One possible explanation would be to consider overcrowding a subclinical stressor. Moberg (2000) defined a subclinical stressor as one that depletes biological resources of an animal without creating detectable change in function such as milk yield, reproduction, or health. However, it leaves the animal unable to successfully respond to additional stressors. Using this model, one can imagine an overcrowded pen of dairy cattle that appears to be meeting most or even all of the commonly used industry performance benchmarks, but at some point, secondary stressor(s) will cause a measurable change in function. We propose that the extent to which the biological reserves are expended by the subclinical stress of overcrowding in any specific herd is a function of the quality of the housing and management routines.

Mac Campbell embraced this model and asked the question of what occurs with chronically overcrowded dairy cows when the secondary stressor is inadequate ration fiber and feed availability (Campbell and Grant, 2016). As a ruminant, little is as important to dairy cattle well-being and productivity as a healthy rumen environment, particularly pH. When comparing stall and headlock stocking density of 100 versus 142% with physically effective neutral detergent fiber (peNDF) of 19 or 21% (8.5 and 9.7% undegraded NDF at 240 h of in vitro fermentation; uNDF240), Campbell and Grant (2016) reported that the extent of subacute rumen acidosis (time that pH <5.8) was up to 2 h/d greater for the main effect of overstocking versus fiber. Furthermore, when cows were fed the same total mixed ration (TMR), cows overstocked at 142% and also experiencing a 5-h feed restriction prior to delivery of the morning ration had up to 9 h/d greater subacute rumen acidosis than those at 100% stocking density with unrestricted feed access. The bottom line was that stocking density and feed restriction had a greater negative impact on rumen pH than dietary fiber characteristics. In fact, this work suggests that the ideal recipe to lower rumen pH would be to: 1) feed a highly fermentable diet, 2) overcrowd the feed bunk and stalls, and 3) feed to an empty bunk.

We have joked that this research does not give the nutritionist a “get out of jail free card,” but it clearly recasts the relative importance of ration formulation and feed-bunk management. *Ration formulation, especially carbohydrates, is critical to maintaining healthy rumen pH conditions, but we now know that how the ration is fed is paramount*.

Overcrowding resulted in lower rumen pH in Campbell’s work, but it did not have great effects on feeding or ruminating time. Like previous research, lying time was significantly reduced, and notably recumbent rumination was depressed at higher stocking density. With overcrowded environments, Campbell (2017) observed a negative relationship between the fraction of rumination that occurs in the free stall (x) and hours that rumen pH is below 5.8 ($y = -20.7x + 21.1$; $r = 0.66$). This result had not been expected but raised interesting questions regarding the potential importance of rumination in stalls to maintaining desirable rumen pH conditions. For the first time, *there was evidence that posture during rumination (i.e., standing or lying) affected rumen pH*. This makes sense given what we know about the relative rates of saliva secretion when the cow is eating, ruminating, or resting, and how sternal recumbency aids the rumination process (reviewed by Grant and Cotanch, 2023).

Management and Milk Components

Based on the positive relationship between rumen pH and greater milk de novo fatty acids and total fat production (Fukumori et al., 2021), de novo fatty acid content of milk fat should serve as a useful barometer of rumen pH conditions conducive to fiber digestion and microbial growth. Barbano has published extensively on the positive relationship between milk de novo fatty acids and output of milk fat and true protein (Barbano et al., 2018) and suggested as much. Published literature demonstrates the positive relationship between higher rumen pH and greater de novo fatty acids, milk fat, and true protein percentage (Allen, 1997; Fukumori et al., 2021; Stone, 2004).

At roughly the same time that Campbell was conducting his work, Melissa Woolpert reported on factors affecting milk de novo fatty acid production for 79 commercial herds in VT and NY (Woolpert et al., 2016; 2017). Higher de novo fatty acid herds produced 17% more milk fat and 14% more true protein than lower de novo herds. Across these herds, the top-five factors that characterized high de novo, high component herds were: 1) dietary fat ≤3.5% of ration DM; 2) dietary peNDF ≥21% of ration DM; 3) lower feed bunk and stall stocking density; and 5) greater feeding frequency of TMR. High de novo herds were 10x more likely than low de novo herds to have feed bunk space ≥45 cm/cow and stall stocking density ≤110%. High de novo herds were 5x more likely to feed TMR twice daily rather than once. Importantly, 65% of the variation in milk de novo fatty acid content among herds was explained by feed bunk space alone. This relates well to earlier work that found greater feed bunk space to be correlated with increased milk yield and milk fat percentage (Sova et al., 2013). We cannot overstate the negative effect that overcrowding may have on milk component production. *Overcrowded cows cannot respond maximally in milk components to the formulated ration.*

Considering Campbell's demonstration that greater rumination in the stalls is associated with higher rumen pH, then cows in competitive feeding environments who are able to achieve more recumbent rumination should have better rumen conditions for fiber digestion and potential for greater de novo fatty acids, milk fat and protein output. In fact, McWilliams et al. (2022) observed that cows with greater ruminating time while lying down had greater DMI and produced milk with more fat and true protein content. To follow up on this work, we conducted a student research project in spring 2023 using Holstein cows that ranged from 3.2 to 6.4% milk fat. Similar to McWilliams et al. (2022) we found a significant positive correlation (R = 0.34; $P = 0.03$) between minutes of rumination while lying down and milk fat percentage. This relationship was significantly stronger than for ruminating, resting, or eating time. *Recumbent rumination, and not simply total rumination time, is critical to a healthy, productive, and profitable herd*.

Forage Quality and the Balance Between Eating and Recumbent Rumination

Eating time between 3 and 5 h/d is typically associated with desirable feeding behavior as reviewed by Grant and Albright (2001). In a cow's ideal environment, she will achieve over 80% of daily rumination while lying down. Hence, the cost of excessive time at the bunk, beyond 5 h/d, is considerable since it directly reduces time available for resting and ruminating. In fact, Jiang et al. (2017) observed an exact daily balance between total chewing time, driven by eating, and resting time. As dietary forage content increased beyond 50% of ration DM, eating time increased markedly, with little impact on rumination. The extra time needed for eating was carved minute-for-minute from resting time, and inescapably from resting rumination. *We propose that maintaining a proper balance between eating time (3 to 5 h/d) and optimal resting (11 to 14 h/d) and ruminating time (8 to 9 h/d) is central to cow productivity and well-being*.

Understanding the fundamental importance of maintaining the balance between eating and recumbent rumination was paradigm shifting. And it begged the question of how forage quality influences the cow's ability to keep eating and resting/ruminating in

balance. Fiber plays a major role in stimulating chewing, both eating and ruminating. Dietary fiber content, source of fiber, particle size, digestibility, and fragility all contribute to the effect of fiber on chewing. Grant and Cotanch (2023) reviewed recent research showing that the primary effect of forage particle size is on eating rather than ruminating in many feeding scenarios. Cows chew to a relatively uniform particle size endpoint prior to swallowing while eating. Consequently, the rumen is populated with a forage particle size distribution that is smaller and more uniform than the TMR. In fact, for many of our commonly fed corn silage-based diets the particles retained on the 8-mm sieve of the Penn State Particle Separator are essentially the same size as the swallowed bolus while eating.

Particle size reduction that occurs during eating requires more chews per gram of feed DM and takes longer for diets that are coarser, higher in forage NDF, and less digestible. We have measured up to a 6-fold reduction in the longest TMR particles prior to ingestive swallowing when we fed cows coarse diets with high uNDF240 (Grant et al., 2018; Smith, 2019). The published literature shows that eating time can be increased by up to an hour when forage NDF is greater, less digestible, or chopped coarser (Grant and Ferraretto, 2018; Grant and Cotanch, 2023). Details are provided in the review by Grant and Cotanch (2023), but the main point is that a system has been proposed that allows theoretical length of cut to be adjusted based on forage maturity, moisture content, and digestibility or fragility to optimize the balance between eating and recumbent rumination. Additionally, an optimal TMR particle size distribution has been proposed to balance eating, ruminating, and resting.

In our approach to particle size, *eating time at the feed bunk is a crucial and overlooked component of forage quality*. Lower quality forage is less digestible and takes longer to eat unless particle size is reduced. Likewise, higher quality forage benefits from longer chop length in most cases. Understanding the fundamental importance of this relationship will be most critical in competitive feeding environments – which characterize many of our commercial herds. We view this as a holistic approach to optimizing the two fundamental components of a profitable dairy farm: forage quality and cow comfort.

Modeling Management: The Holy Grail?

Publications by Grant and Tylutki (2010; 2011) for the first time proposed that time budget analysis should become a routine and important part of DMI prediction and ration formulation. The feeding environment is comprised of both physical and social components that modulate feeding behavior and feed intake in dairy cattle. Currently, key components of the physical environment such as temperature, humidity, wind speed, and so forth are inputted into the model during ration formulation. But, effectively incorporating social and management factors such as time budgets, stocking density, and feed availability has proven challenging. Nonetheless, nutrition models need to incorporate management inputs. Feeding behavior and intake are dramatically affected by time available to eat, forage NDF characteristics, notably particle size, and stocking density to name a few.

To date, the best effort has been the management model created as part of Michael Miller's Ph.D. dissertation (Miller et al. 2020; detailed equations provided by Miller, 2020). The model remains a work-in-progress, but initial field experience suggests that it has usefulness. It has been implemented in AMTS version 4.18 as a recipe tool called the "management model."

The model was designed to allow input of commonly measured farm variables such as stocking density and milking time to assess the effect of management decisions on DMI, milk production, and behavior. The model is divided into five sections: 1) behavioral time budget adapted from the original model by Grant and Tylutki (2011); 2) stocking density calculation; 3) eating time prediction; 4) DMI prediction; and 5) physically effective uNDF240 (peuNDF240) adjustment to DMI. The time budget analysis provides time available for eating plus resting, and eating time is predicted from NDF, peNDF, body weight, milk yield, and feeding frequency. Once time available for rest has been calculated, it is adjusted based on stocking density of stalls or manger, depending on whether the barn uses headlocks or post-and-rail. Then, we use a relationship between resting time and milk yield, based on a review of published data to predict fat-corrected milk production. The fat-corrected milk value, together with body weight and week of lactation, is used to predict DMI using NRC (2001) equation. Although this approach backs into a DMI prediction, it seems to be the best approach for now since no reported relationship between stocking density and short-term DMI has been reported.

Three main limitations remain: 1) rigorous model validation; 2) adjustments for parity effects; and 3) incorporation of feed availability. Although we know that parity and feed availability are important, a lack of data hampers further model development. The model is most sensitive to milking time and stocking density which makes sense given how important they are on farm. As part of model development, Miller (2020) reviewed the published literature and updated equations relating stocking density with lying time and lying time with milk yield. Although not a main part of the model, an intake adjustment based on the relationship between peuNDF240 and DMI was also incorporated to take advantage of the database we have built comparing uNDF240 and peuNDF240 and their relationship with DMI and energy-corrected milk (Grant et al., 2018; Miller et al., 2020; Farricker et al., 2022). It is conceivable that this component in the future could also incorporate the interaction between rumen fermentable starch and peuNDF240 (Smith et al., 2020). Stay tuned.

We Need to Push Forward!

The amount of cattle management research conducted over the past twenty years is mind boggling. When reviewing the many management factors affecting cow performance and health, few are surprising. *What is surprising is the sheer magnitude of the cow responses to an improvement in comfort*. Furthermore, it is amazing how a few factors rise repeatedly to the top as essential for a low-stress management environment: 1) time available for eating, resting, and ruminating; 2) managing stocking density and overcrowding; 3) ensuring feed availability 24/7; and 4) resting area comfort (e.g., deep bedding). Regardless of the management system – free-stall and parlor, automated

milking system, tie stall, pasture, or whatever evolves – we need to understand how to accommodate natural cow behavior. Any sustainable dairy system will consider environmental, welfare, and societal concerns. And from a profitability perspective, there is little doubt that cow comfort economics will remain compelling!

Research needs to continue that builds on our new understanding of forage quality and cow well-being. How forage characteristics, housing, and management environment affect each cow's ability to balance eating with recumbent rumination needs to be a focus going forward. As advances in nutrition models occur, we need to implement a functional model incorporating physical and social components of the cow's environment. Time budgeting should become a routine part of ration formulation.

As behavior research moves toward answering much-needed questions that cross-cut society, such as cow-calf separation, we cannot forsake research aimed at enhancing the productivity and well-being of cows in commercial management systems. So, we return to the original assertion of this paper that I believe captures the essential importance of our work at Miner: "*cows that aren't rushed while eating, have the freedom to lie down and ruminate, and can strike the correct balance between eating and recumbent rumination, will have optimal rumen conditions for fiber digestion, microbial growth, and healthy production of more milk components."*

Our management research at Miner reaches back to Jack Albright's pioneering work in cattle behavior; his recognition that the need to accommodate natural cow behavior would become an essential component of successful herd management. After briefly reviewing two decades of management research at Miner Institute and looking to the future, I conclude that applied dairy management research must continue with the goal of "unleashing every cow's inner Ellen!"

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