Viewing a Role for Isoacids in Dairy Nutrition Through a New Lens

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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.

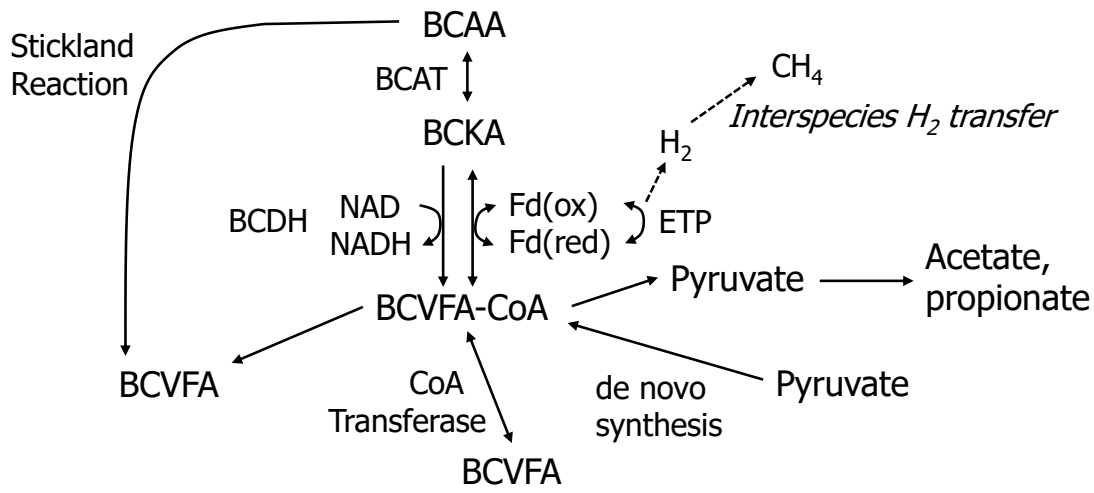

Figure 1. Likely mechanism by which isoacids are used by ruminal bacteria. BCAA = branched chain amino acids, BCVFA = branched chain VFA, BCKA = branched chain keto acid, BCAT = branched chain aminotransferase, BCDH = branched chain keto acid dehydrogenase, Fd = ferredoxin that is oxidized or reduced,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

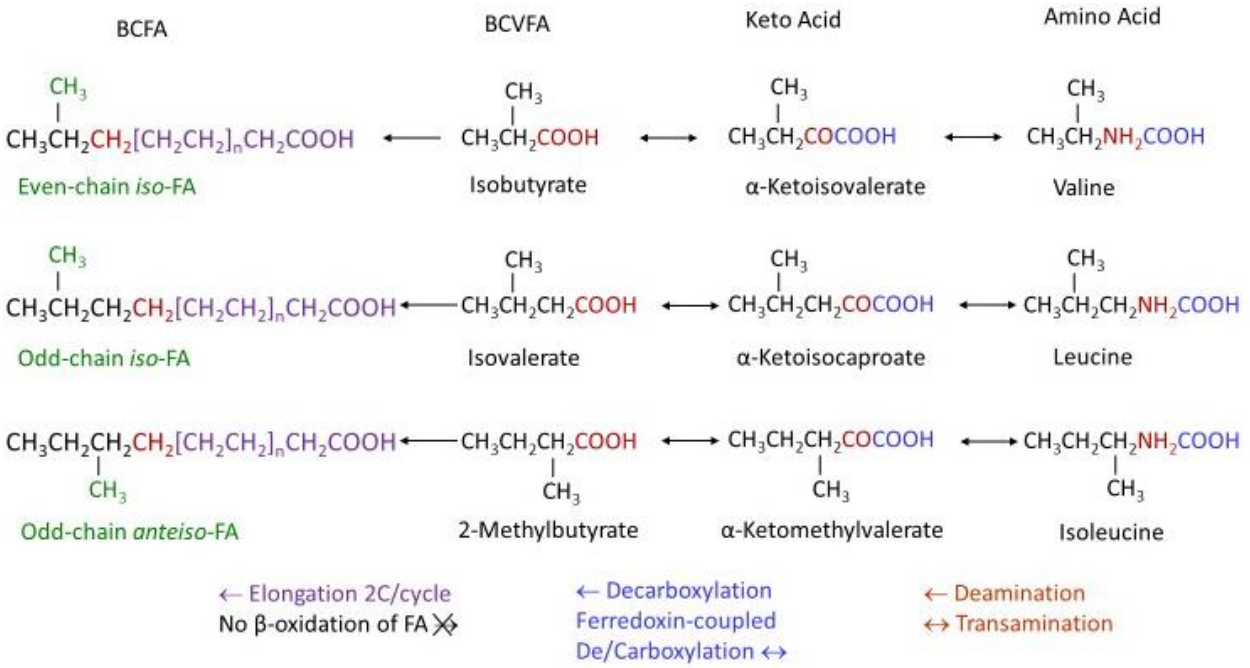

Figure 2 Unlike with aerobic cells, β-oxidation of FA is unlikely to shorten these chains in the anaerobic rumen. Anteiso configurations are on the opposite side of the BCFA than iso methyl groups and are more fluidizing.

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.

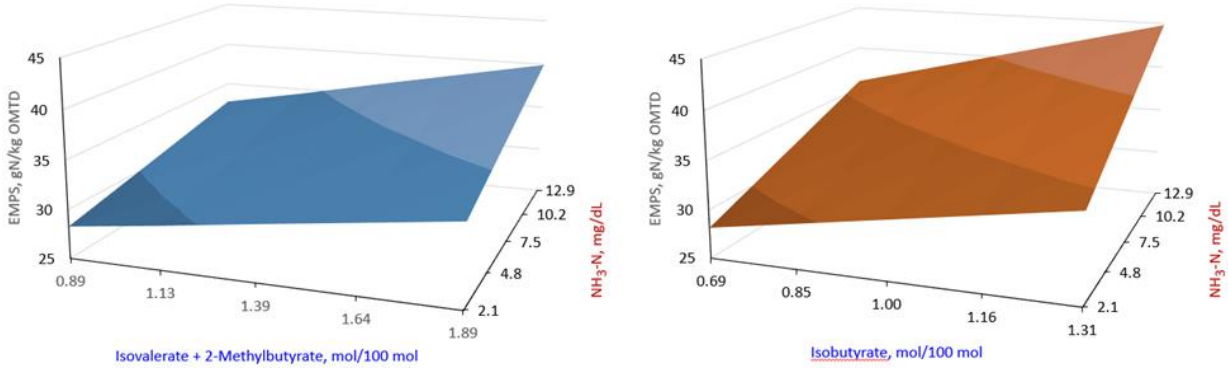

Figure 3. The equations from Roman-Garcia et al. (2016) to predict efficiency of microbial protein synthesis (EMPS; Y axis) were truncated to the mean ammonia concentration of 12.9 mg/dL projecting downward (Z axis) and shown for increasing isovalerate (actually a mixture of IV and 2MB) or isobutyrate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

References

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