Role of Isoacids to Enhance Rumen Function

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Abstract

The 'isoacids' or branched-chain volatile fatty acids **(BCVFA)** have been known for decades to be required for the cultured representatives known to degrade cellulose and even some other members of the microbial community. We will discuss how ensuring that the primary cellulolytics are provided with an optimum supply of BCVFA should not just increase fiber digestibility but improve the balance of the entire microbial community. Previously published work demonstrated improved fiber degradability, efficiency of microbial protein synthesis, and/or a post-ruminal effect. Supplementing BCVFA should be revisited as we lower safety factors for rumen-degraded and -undegraded protein in the diets of dairy cattle that are far higher yielding and more efficient than they were when isoacids were previously marketed. Our goal is to explain how isoacids can enhance rumen function so that dairy nutrition advisors can decide in what ways to use this product in dairy diets.

Introduction

Similar to forage source/quality being the basis for dairy rations for sustainable production, the abundance and activity of the fibrolytic (i.e., fiber-degrading) microbes in the rumen is the basis of a stable microbial community in the dairy cow's rumen. In particular, the fibrolytic microbes are intended to work as a consortium that includes other groups of microbes. We need to distinguish the context of fibrolytic microbes in a complete community and extend beyond the expectation that they degrade cellulose independently, but we still need to start with the cellulolytics to understand a role for isoacids to enhance rumen function.

We have known for decades that pure cultures of cellulolytic bacteria require certain branched-chain volatile fatty acids **(BCVFA)** as growth factors, whereas few of the culturable strains of amylolytic and hemicellulolytic bacteria were documented to require BCVFA. Later studies progressed from evaluating a requirement to evaluating growth rate. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus* are prominent cellulolytics that require BCVFA for optimal growth on cellulose. They also contribute to the degradation of hemicellulose, even though they do not even use its resultant sugars. They share oligosaccharides from hemicellulose and even cellulose in exchange for BCVFA from branched-chain amino acid **(BCAA)** degradation by more generalist bacteria (Koike and Kobayashi, 2009). Moreover, there are many studies in which adding a nonfibrolytic partner to a pure culture of a cellulolytic bacterium improved digestibility of cellulose and probably would have for hemicellulose had it been measured more often. Many of those culture-based studies have used a

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purified source of cellulose or hemicellulose that does not represent the complexity or diversity of substrates available to cows (discussed later).

When we feed the cow (or "feed the rumen"), we are providing a blended combination of feeds that is intended to meet those microbes' needs without overfeeding grain to limit fiber degradation or to limit efficiency of microbial protein synthesis **(EMPS)**. More specifics are included in a recent review (Firkins, 2021). For this paper, we consider that we are feeding cows based on software that assumes no diurnality in the daily supply vs requirements computations, yet feeding dynamics probably influence microbial efficiency by relating BCVFA availability with cellulolysis at least for part of the feeding cycle. We also are aiming to limit excretion of nitrogen or emission of methane, yet such actions might lower rumen microbial efficiency. The BCVFA and ammonia concentrations vary in different patterns after feeding, and decreasing rumen-degraded protein **(RDP)** lowered abundance of cellulolytic bacteria but also diversity of the entire population (Belanche et al., 2012).

Many of us remember IsoPlus® from the 1980's in which isobutyrate **(Ibu)**, 2-methylbutyrate **(2MB)**, or isovalerate **(Ival)** were combined with valerate. The first three BCVFA have a methyl branch ("iso" in chemistry); although valerate is a straight-chain VFA, it was included because it was deemed a growth factor for some pure cultures. Our work (Roman-Garcia et al., 2021a) and that of others suggests valerate is used as directed but that its concentration is never actually limiting in the rumen of dairy cattle, so it will not be discussed further. Similar conclusions were made for other BCVFA work in vitro (Gorosito et al., 1985), but important caveats need to be considered. First, they used fluid-phase bacteria, which will limit the response (there will be few of the BCVFA-requiring cellulolytics in that fraction). Second, evaluating concentration per se ignores that researchers control the proportion of substrate:medium, so in vitro concentration should not be directly compared with in vivo values. Another caveat is that 2MB and 3-methylbutyrate (i.e., Ival) are so similar chemically that they have been known for decades to coelute in standard procedures unless modified (Dehority et al., 1967). Because of the misguided notion that 2MB is a minor contributor (sadly, even ignored in our previous OSU studies), the slight statistical preference for Ival over Ibu in our meta-analysis (Roman-Garcia et al., 2016) is expected currently to result from the 2MB coeluting with Ival. Because of the paucity of direct evidence for Ival separate from 2MB in dairy studies, direct responses with cows are challenging to predict. Therefore, our goal is to extend from the older literature to predict the role for isoacids in today's dairy cow by explaining the mechanistic role of these isoacids to enhance rumen function.

What is Fiber and What Limits its Degradation?

As nutritionists, we think of fiber as NDF or maybe cellulose, hemicellulose, and lignin. Pectin is often downplayed because it is difficult to measure directly and is solubilized in neutral detergent solution. OSU researchers defined a more appropriate way is to measure residual organic matter (sometimes called nonstarch-nonfiber carbohydrate, which includes soluble fibers) for digestibility studies with dairy cattle (Tebbe et al., 2017). Extension of this work is sorely needed to assess limitations for digestibility of soluble fiber. With all of the different forages at different maturities and all of the fibrous byproducts we feed, we need to think of fiber more holistically than just "NDF" anyway. A greater understanding of the diversity of insoluble and soluble fiber can now be

integrated with techniques better characterizing the diverse microbial consortia, particularly with respect to more diverse fiber components and to relate to enteric methane production (Kelly et al., 2019).

Cellulose degradation has traditionally been assumed to be limited by surface area (Russell et al., 2009). More exposed surface area is associated with improved forage quality, processing, and ruminal processes, such as rumination, that influence the fragility of forage (i.e., ease of particle breakdown). Again, these processes could be integrated with emerging evidence of the diversity of carbohydrate active enzymes used to degrade these diverse fibrous constituents (Naas and Pope, 2020). Key components include carbohydrate binding domains needed to get enzymes in close proximity to their fibrous substrates. Because the main cellulolytics are not motile, these binding domains apparently increase in importance as substrate diminishes and the void widens between remaining degradable fibers over the course of particle's expanding colonization. Our recent finding that BCVFA likely stimulates the interaction between the nonmotile cellulolytic specialist *F. succinogenes* with its motile noncellulolytic *Treponema* partner (Roman-Garcia et al., 2021b) further supports the need for cellulytics to be in close proximity to their substrate.

Cellulose binding modules can be assembled into larger complexes and with more complicated strategies for efficiently degrading cellulose (Naas and Pope, 2020). *Ruminococcus flavefaciens* has a classic 'cellulosome' with up to 14 different cellulolytic enzymes assembled across 4 different dockerins (proteins connecting the enzymes into a complex analogous to docks connecting ships in a marina). Although closely related, *R. albus* has a simpler extracellular complex, yet still relies heavily on cellulose binding modules. In contrast, *Fibrobacter succinogenes* secretes outer membrane vesicles that are thought to help prepare it for colonizing fibrous particles by pitting the fiber particle. This bacterium does not have cellulosomes or its components. Instead, it appears to contort its cells to colonizes and degrades fiber. Like the cellulolytic ruminococci, it also helps degrade hemicellulose, even though it does not even use many of the resultant sugars. These critical processes for these three best characterized cellulolytics all depend on proper membrane function (discussed subsequently).

Subsequent metagenomics tools are assigning polysaccharide utilization loci to bacteria. Many of these are best characterized for starch hydrolysis. *Ruminococcus bromii* is a prominent starch user that requires Ibu or 2MB and probably proliferates with more starch or lower rumen pH (Roman-Garcia et al., 2021b). Less is known of the rumen strains compared with those from the human gut, where they are known as a major keystone degrader of resistant starch. They apparently have 'amylosomes' (paralleling cellulosomes) like the better studied human gut relatives except that the rumen strains might be more reliant on uptake of oligosaccharides than on free sugars (Mukhopadhya et al., 2018). These starch degraders also probably encode transporters for short oligosaccharides from fiber degradation, including oligosaccharides produced from degradation of cellulose or hemicellulose. Ideally, they probably work in coordination with the cellulolytics, even while they compete for common resources, such as BCVFA. Although the purely hemicellulytic bacteria that lack appreciable cellulase might not require BCVFA, many of the cellulolytics have diverse and active hemicellulases (Moraïs and Mizrahi, 2019; Terry et al., 2019). Thus, in contrast with co-culture studies (many hemicellulolytics can't yet be cultured), metagenomics techniques are even supporting webs of poorly researched members

and substrates (Kelly et al., 2019). Thus, the food web existing between groups of microbes, including those poorly characterized, probably depends directly or indirectly on primary cellulolytics, which do require BCVFA. Their close partners might (e.g., *Treponema*) or might not (e.g., *Selenomonas*) directly require BCVFA but likely are stimulated by adequate BCVFA by virtue of interaction with cellulolytics.

What does a potential 3 to 5% unit improvement in NDF digestibility (as we have typically seen in our in vitro studies) mean for dairy cattle? Simply put, the more efficient fiber degraders are better able to degrade the more diverse or more recalcitrant fiber components in diets before those particles pass out from the rapidly turning over rumen of the high producing cow. A more fully colonized particle also washes out more microbial protein for the cow. Less simply, improved mechanics of microbial fiber degradation probably is most likely to increase the rate of degradation. A faster rate of degradation probably increases either ruminal digestibility or would maintain similar digestibility by indirectly promoting a faster passage. A faster degradation could mean that plant particles break down and lose their integrity (gaining surface area to increase rate of degradation). However, this same increased rate of particle disintegration allows fermentative gas to escape these fibrous particles more quickly, thus promoting particle sinking to, and increased passage rate from, the reticulo-omasal orifice. Either greater digestibility or a comparable digestibility but with less rumen fill should allow a higher intake of rumen-degradable fiber. Our meta-analysis documented this paradox that NDF digestibility is positively associated with DMI because more digestibility seems to allow a higher intake rather than a higher intake would increase passage rate and therefore decrease fiber digestibility (White et al., 2016). A 4% unit improvement in NDF digestibility should increase DMI about 1.5 lb/day or milk by 2.2 lb/day (Oba and Allen, 1999). Conversely, if cows are in late lactation, such an improvement in fiber digestibility (and greater digestible energy) should allow the same milk but with lower feed intake. Either way should improve feed efficiency.

Role of Cellulolytic Bacteria in the Balanced Ruminal Microbial Community

Just as a replacement heifer represents your farm's future, the so-called "keystone" cellulolytics in the cow's rumen today also represents its community structure tomorrow. If cellulolytics were limited in abundance by availability of needed nutrients, such as BCVFA, then their abundance at each successive meal could decline on its own. Because the progressively 'next' meal is increasingly colonized by microbes that are not as efficient at degrading fiber but is not as limited by BCVFA, eventually either lower fiber digestibility or lower intake would decrease feed efficiency. Fortunately, the cow's rumen microbial community tends to be resilient (i.e., reverts back to the original community structure). However, we want resiliency when it maintains an optimal microbial community structure, but of course, not when it maintains a less-than-optimal community structure. I have borrowed the "rock-paper-scissors" analogy of Israeli researchers (Moraïs and Mizrahi, 2019) in which there is competition for substrate, but each of the three analogous niches average out for a balanced total community (Firkins, 2021). On average, the rock, paper, and scissors all are just as likely to win the game. Primary colonizers share resources with secondary colonizers, which share back with primary colonizers to have an overall efficient consortium unless there is an imbalance, such as excess starch relative to effective fiber or RDP. That is, the rock increasingly wins against the cellulolytic scissors because the paper gets diluted out.

Rather than trying to glean every last portion of degradable carbohydrate as in a gestating beef cow fed poor quality forage, a high producing dairy cow needs to optimize its degradation of fiber and its passage rate simultaneously to maximize intake of rumen-degraded fiber. As explained previously, optimized intake of rumen-degraded fiber allows high feed intake during at least part of the feeding cycle unless high starch in the diet limits intake by chemical factors, such as high propionate oxidation in the liver. As documented through Mike Allen's extensive research at MSU (Allen et al., 2019), optimizing NDF flux through the rumen involves both plant factors (e.g., forage maturity) and animal factors (e.g., animal demand or rumination efficiency). When there is a drive by high-producing cows to eat more, fill restriction is more important, and factors related to faster particle breakdown stimulate high feed intake.

At the 2021 Tri-State Dairy Nutrition conference, we described additives to help smooth transitions toward increased starch fermentability to limit its potential inhibition of fiber degradation. Along with improving forage quality and other managerial factors, these are some practices we can use successfully. However, Firkins (2021) also discussed the rather surprising ability to assign heritability coefficients to rumen microbial species. Some heritable taxa have a critical function, such as the keystone fibrolytics that require BCVFA. Others assigned as heritable have a less clear function that, if better understood, could help us better close the gap on how to improve feed efficiency for dairy cattle.

Efficiency of Microbial Protein Synthesis

We can increase the amount of microbial protein supply to the cow by increasing the microbes' main source of energy -- rumen-degraded carbohydrate. However, this carbohydrate should be a proper blend of fiber and starch to minimize the challenge that more starch limits fiber digestibility (Ferraretto et al., 2013). The microbes also use dietary sugars, and in some diets, a lot of soluble fiber not measured in NDF, but there is little known to summarize how these influence EMPS. For some reason still not well understood, supplemental sugars sometimes improve NDF digestibility (Oba, 2011). In that case, one might expect an improvement of EMPS if there is a more balanced consortium. However, supplementing sugars decreased ammonia or BCVFA concentration in some in vitro studies, potentially supporting the need for more RDP or perhaps its components (such as BCVFA) with increasing supplementation of sugars (Firkins et al., 2006).

The equation in NASEM (2021) predicting microbial supply is structured as EMPS:

$$\text{Microbial N (g/d)} = \frac{(101+82.6\ (RDP\ intake)}{(0.094(RDNDF)+0.027\ (RDS)}$$

Where RDP, rumen-degraded NDF (RDNDF), and rumen-degraded starch (RDS) are all in kg/day.

The numerator has microbial N's main precursor, RDP. About 15% of the microbial N is from transfer of blood urea in dairy cows, but RDP contributes to blood urea synthesis. The denominator predicts the main source of energy from RDNDF and RDS. The denominator's component of the equation is not very sensitive to changes in NDF or starch in the diet because increasing starch was associated with decreasing NDF digestibility. Thus, with greater intake of RDNDF and RDS, a greater intake of RDP also is needed. However, by virtue of the Bayesian

parameterization to include RDP's coefficient in the numerator, the model is particularly sensitive to RDP because it has a linear slope. Of course, RDP can provide a smaller but significant amount of energy for microbes independent of rumen-degraded carbohydrate; hence, even without an increase in rumen-degraded carbohydrate, more RDP is modeled to increase microbial N supply (and therefore to amino acids digested in the small intestine). All attempts to "flatten the curve" (i.e., make the equation asymptotic) for RDP did not fit well. To prevent overreaching beyond the database, the RDP response is truncated at 12% RDP. Either way, improved rumen-degraded NDF using isoacids should increase EMPS beyond what would be predicted by this equation; moreover, we are evaluating whether isoacids could substitute for a portion of RDP. Finally, because of limited information, we could not include parameters for residual OM (i.e, from soluble fiber not recovered in NDF), but the coefficients account for this fraction to the degree to which diets being assessed are similar to the average residual OM in the database used to derive the equation.

Because RDP is rarely measured, we included ammonia and BCVFA as variables related to EMPS (Roman-Garcia et al., 2016). Figure 1 illustrates the predicted EMPS with increasing 'isovalerate' (i.e., the sum of Ival plus 2MB) or Ibu at their respective means ±1 standard deviation. Figure 1 also illustrates predicted EMPS as ammonia-N declines from its mean (12.9 mg/dL) minus 2 standard deviations. Above the mean, the relationships are quadratic and reverse directions. However, even the mean of 12.9 mg/dL of NH3-N should be adequate for rumen microbes; increasing beyond that would likely indicate some factor besides RDP was limiting EMPS in the database (researchers often would overfeed protein intentionally to prevent it from being a confounding variable). Figure 1 clearly shows the dual importance of increasing BCVFA and NH3-N to optimize EMPS. Since that meta-analysis, our more current research has clearly demonstrated that the response to 'isovalerate' was most likely a result of its hidden 2MB contribution (Roman-Garcia et al., 2021a).

BCVFA or BCAA for Bacterial Protein Synthesis

We have known since the 1950's that some prominent cellulolytic bacteria in the rumen, *Ruminococcus* and *Fibrobacter*, require BCVFA. Dehority et al. (1967) noted that Ival is much less important (or not at all for *Fibrobacter* strains studied) than Ibu and 2-MB, with the latter BCVFA often substituting for one another, whereas in one strain of *Ruminococcus*, he noted that 2-MB was highly stimulatory in the presence of Ibu. He also noted that valerate's requirement was lessened by providing acetate, which agrees with our studies.

More contemporarily, metagenomics has expanded our information on BCVFA. Because of a lack of one or more genes used in the pathways to synthesize all 3 BCAA, cellulolytic *Ruminococcus* and *Fibrobacter* bypass the normal BCAA synthetic pathway altogether by reductive carboxylation of BCVFA produced by other bacteria (Figure 2). Standard pathways described for enteric bacteria to interconvert BCAA and BCVFA are not annotated in the typical rumen microbes (Trautman et al., 2020). We assume that this reductive carboxylation pathway of BCVFA to BCAA increases when we feed BCVFA because it should increase diffusion or passive transport of BCVFA inside cells. If the cellular free BCAA concentration is relatively low (as would occur with rapid protein synthesis) and the ferredoxin-linked cofactor is highly reduced (as would occur with high fermentation rate), then BCVFA would be converted to BCAA. The 3 BCAA are almost

20% of the total bacterial protein (Sok et al., 2017).

As shown in vitro, preformed BCAA have a critical role to maintain microbial protein synthesis (Atasoglu et al., 2004) so long as they are balanced (Kajikawa et al., 2005). The aromatic amino acids also seem to have a critical role, as those authors explained. That said, when expressing the AA in alfalfa hay, corn silage, and soybean meal relative to respective AA in rumen bacteria (Patton et al., 2014), preformed Leu and phenylalanine should be more available than Val and especially Ile relative to the composition of bacterial protein. Leucine is higher concentration in bacteria than are the other AA, explaining the high recovery of ^{13}C-Ival in Leu (presented by K.E. Mitchell at this conference). Moreover, the keto acid of Val is routed to synthesize pantothenic acid and Leu (Figure 2). Pantothenic acid is, of course, needed to metabolize BCAA and even for production of the other VFA. Because 2MB produced in the cow (or dosed) is racemic, up to half of the 2MB is poorly used. The other BCVFA are not racemic. Our high^{13}C recovery from 2MB, despite is racemic nature, in bacterial Ile supports our consistent identification of this BCVFA as being most critical to NDF digestibility compared with Ibu and especially Ival (Roman-Garcia et al., 2021a).

The BCVFA precursors probably are far more neutral on cell metabolism compared with their respective BCAA. For example, researchers have dosed single amino acids one at a time or removed them one at a time from a standard complete mix. Dosing only Ile depressed growth rate of ruminal bacteria in batch culture (Kajikawa et al., 2002). Adding Leu alone also inhibited growth but corrected growth inhibition by Ile; adding Val also corrected the Ile growth inhibition, but Val did not inhibit growth when added alone (Kajikawa et al., 2005). Yet, all 3 BCVFA in balance improved bacterial growth. These effects should be expected based on their common metabolism.

We assume there should be a balance of these BCAA and to avoid an imbalanced supply of especially Leu and Ile. That Val alone did not depress bacterial growth might explain why adding Val (decarboxylated to Ibu) was responsive for milk production (Hultquist and Casper, 2016). Leucine was catabolized to a greater extent than were Ile or Val (Atasoglu et al., 2004). Although all 3 BCAA can influence gene expression in bacteria, an excess of Ile might be more inhibitory to transcription of many critical anabolic enzymes or operons (series of enzymes in a pathway) in laboratory strains of Gram-positive bacteria (Kaiser and Heinrichs, 2018). Assuming a similar response to rumen bacteria, feeding the BCVFA vs their parent BCAA would remove potential BCAA imbalance, especially for Leu and Ile.

When the BCAA are converted to BCVFA, some bacteria most likely are deriving ATP by chemical reactions called oxidative decarboxylation (best shown in enteric bacteria and pathogens) or Stickland reactions (best shown in clostridia) that are assumed to apply to the rumen bacteria. However, the conversion of BCVFA back to BCAA is a minor ATP cost compared with potential feedback responses that might lower EMPS because of potential imbalanced accumulation of a BCAA. The direct uptake of BCVFA still spares energy that would be lost when BCAA are synthesized compared with pyruvate routed to VFA (Figure 2). We need to remember that sparing the BCAA via its BCVFA precursor allows the synthesis of all of the enzymes needed to produce those BCAA from pyruvate. Hence, BCVFA are readily used by noncellulolytic bacteria that could otherwise make their own BCAA. For example, the predominant ruminal bacterium, *Prevotella*

ruminicola, does not require BCVFA and yet will readily convert considerable amounts of Ival into Leu (Allison et al., 1984). Finally, uptake of BCVFA to produce BCAA or BCFA allows the glucose to route to many other cellular building blocks not shown in Figure 2. Although not verified, further research needs to corroborate and expand on the potential that increased Ival (or other BCVFA) can increase expression of anabolic enzymes stimulating protein synthesis, as suggested for *P. bryantii* (Trautman et al., 2020).

Branched-Chain Volatile Fatty Acids for Bacterial Lipids

The BCVFA also are elongated to branched chain fatty acids (BCFA; Figure 2). If the bacterium cannot produce BCAA, then it also cannot produce the BCVFA precursor for branch-chain fatty acids **(BCFA)** because the lost genes in BCVFA-requiring bacteria are at enzymatic steps after pyruvate but before the precursor for BCVFA. BCFA are well known to play key roles in maintaining cell membrane integrity of all anaerobes, including those in the rumen. Cell membranes are required to serve as barriers, of course, but they also must maintain proper 'fluidity' to allow transport of sugars, amino acids, and ions through carriers or channels along with membrane movement needed for cell division. The methyl branch ('iso') in BCFA maintain enough distance between the FA in the phospholipid membrane to allow this fluidity, yet the BCFA are converted to aldehydes **(BCALD)** in plasmalogens that also seem to stabilize the membrane.

Our research with ^{13}C-labeled BCVFA has documented their importance for BCAA synthesis, which is the main sink for ^{13}C because cells are so high in BCAA (Sok et al., 2017). However, we have also documented the importance for 2MB to produce BCFA and from the BCFA to BCALD (top of Figure 2). Straight-chain fatty acids and aldehydes also are made, of course, but the critical response seems to be from these BCALD as a potential mechanism whereby supplemental BCVFA can improve NDF digestibility. Fatty aldehydes, especially 16:0, iso-14:0, and anteiso-15:0, seem to be inserted as plasmalogens (bottom of Figure 2) in which the vinyl (double) bond helps to stabilize the membrane both structurally and from damage by oxygen. Nutritionists are well versed in how vitamin E is inserted into membranes of animal cells. Similarly to how vitamin E interrupts the autocycle of lipid peroxidation from oxygen radicals, the vinyl (double) bond in plasmalogens also interrupts this oxidation cycle (Jackson et al., 2021). Kelly Mitchell's preliminary data suggest that BCALD are only about ~6% of the sum of BCFA +BCALD, but the ^{13}C recovery from dosed BCVFA was ~25% of the total recovery in that combined lipid fraction because BCALD were about half of the total aldehydes.

Polyunsaturated and medium chain fatty acids can inhibit rumen bacteria, particularly those that degrade fiber, when supplemented in high amounts in the diet or provided in bolus forms (Jenkins, 1993). In lower amounts, though, fat might be stimulatory to rumen microbes by direct insertion into membranes and thus sparing carbon that could otherwise be routed to VFA production (Hackmann and Firkins, 2015). In contrast with expectations based on studies with high amounts of free oil (Jenkins, 1993), at typical feeding levels, unsaturated fat had minimal effect on NDF digestibility (Weld and Armentano, 2017). Adding a supplemental source of palmitic acid increased NDF digestibility (dos Santos Neto et al., 2021), potentially more so with increasing addition of oleic acid (de Souza et al., 2021). In fact, bacteria (probably also rumen bacteria) can incorporate palmitic and oleic acids in their

membranes to make them more firm or less firm, respectively. There is less information on stearic acid. Our preliminary data reported at this conference suggest that increasing forage in the diet increases BCVFA uptake into BCAA and lipid fractions. In high forage diets, we noted increased BCVFA conversion to BCFA and BCALD in lower forage diets, but adding corn oil (approximately 60 and 25% linoleic and oleic acids) seemed to increase the need for BCVFA conversion to BCALD. Our preliminary data suggest that adding corn oil increased oleic acid in bacteria and compensated by decreasing even-chain iso BCFA and BCALD. That the odd-chain BCFA and BCALD were not decreased suggests a vital importance regardless of preformed oleic acid. As described previously, maintaining membrane integrity is likely very critical for cellulose binding domains. Although rarely studied, switching from cellobiose to cellulose as substrate increased the uptake of oleic acid to increase membrane fluidity by *Eubacterium cellulosolvens* (Moon and Anderson, 2001). Details are unclear, but the conditions for evaluating this shift did not consider BCFA from BCVFA precursors. Because no oleic acid was reported (Saluzzi et al., 1993), maintaining a relative ratio of BCFA to BCALD from BCVFA primers seems important to the cellulolytic *Ruminococcus* and *Fibrobacter*. Our research is attempting to fill these gaps with mixed bacteria.

Isoacids for Dairy Cows

Numerous papers were published from the 1980's (Andries et al., 1987). Those authors noted 4 to 8% increase in milk production. Long-term lactation studies (Papas et al., 1984; Peirce-Sandner et al., 1985) documented that some of the individual university trials within their reports had improved feed intake and resultant milk production for an isoacid product typically in a ratio of about 1.4, 1.0, and 1.0 of Ibu, Ival, and 2MB on a molar basis. Other university trials with somewhat different diets or herd dynamics maintained or improved milk production with lower feed intake. They also suggested both ruminal (i.e., increased fiber digestibility or microbial protein supply) and post-ruminal responses to explain their results. As readers might recall, one particular criticism of this product was its smell, whereas Zinpro's product is much less volatile.

In our in vitro studies, we have noted a consistent 3 to 5% unit improvement in NDF digestibility when dosing BCVFA. The increased NDF digestibility in continuous culture was related to an increased abundance of two well known consortium members, *Fibrobacter* and *Treponema* (Roman-Garcia et al., 2021b). As explained previously, they both require either/or Ibu and 2MB. Microbial N outflow was not increased in that study. In contrast, in another study (Kelly Mitchell, unpublished data), adding BCVFA increased NDF digestibility and both amount and efficiency of microbial protein synthesis. In this case, there were no differences for relative abundance of any microbes, so we assume that a consistent increase in total abundance without a shift among individual bacterial populations resulting from BCVFA supporting a well-balanced microbial consortium. Preliminary work also shows that this increased microbial N transferred to an increased supply of most of the amino acids in bacterial protein but especially to the BCAA, which increased by about 10%. Our conclusion that BCVFA stimulated protein synthesis in a more balanced consortium is supported by BCVFA significantly increasing the diversity of bacterial populations in lactating cows (Lee et al., 2021).

Isoacids (80 g/day of all 3 BCVFA in equal proportion) increased DMI and production of milk and milk components in a long-term lactation study (Wang et al., 2019). This group

has several publications (many with steers) that report increased abundance of cellulolytic bacteria, increased fiber degradation activity in situ, and increased urinary excretion of purine derivatives (an index of rumen microbial protein production) with supplementation of BCVFA. Total tract digestibility of most nutrients (including ether extract) increased at a decreasing rate with increasing Ibu supplementation (Liu et al., 2009). Increasing supplementation of all 3 BCVFA improved NDF digestibility (Liu et al., 2018). Those authors reported an increase in yield of milk fat (especially short- and medium-chain fatty acids representing the fatty acids produced in the mammary gland). This increase corresponded with increased gene expression of enzymes involved in de novo fatty acid synthesis in mammary gland. When RDP apparently was not limiting in lactating dairy cows, we did not see a benefit in NDF digestibility (Copelin et al., 2021), but the isoacids helped prevent milkfat depression apparently by stimulating milk fatty acid synthesis in the mammary gland (Lee et al., 2021). In another OSU study with 60 Jersey cows (Mitchell et al., unpublished data; reported at 2020 ADSA), feed efficiency improved by over 5% when Ibu and 2MB were supplemented to Jersey cows.

Conclusions

Research is evolving to support a consistently improved fiber digestibility from isoacid formulation. Valerate appears not needed, and isovalerate is either not needed or needed at lower amounts than the other BCVFA in diets with a lot of corn protein (high in Leu, the precursor of Ival). We have provided mechanistic interpretations why these results should be expected as we compile more cow data to coordinate with expectations based on our in vitro results. Our goal is to find out how to coordinate isoacid supplementation with the RDP supply in dairy diets. We will be evaluating whether the increase in BCAA supply in microbial protein in continuous culture is repeated when measuring omasal bacterial flow in dairy cattle; if so, increased BCAA supply would likely influence milk production and partition of nutrients to peripheral tissues. There likely is a post-absorptive role for BCVFA (e.g., in the mammary gland's fatty acid synthesis gene expression), but the bulk of our current results identifies a role to enhance rumen function and thereby feed efficiency.

References

Allen, M.S., D.O. Sousa, and M.J. VandeHaar. 2019. Equation to predict feed intake response by lactating cows to factors related to the filling effect of rations. J. Dairy Sci. 102:7961-7969.

Allison, M.J., A.L. Baetz, and J. Wiegel. 1984. Alternative pathways for biosynthesis of leucine and other amino acids in Bacteroides ruminicola and Bacteroides fragilis. Appl. Environ. Microbiol. 48:1111-1117.

Andries, J.I., F.X. Buysse, D.L. de Brabander, and B.G. Cottyn. 1987. Isoacids in ruminant nutrition: Their role in ruminal and intermediary metabolism and possible influences on performances--A review. Anim. Feed Sci. Technol. 18:169-180.

Atasoglu, C., A.Y. Guliye, and R.J. Wallace. 2004. Use of stable isotopes to measure de novo synthesis and turnover of amino acid-C and -N in mixed micro-organisms from the sheep rumen in vitro. Brit. J. Nutr. 91:253-261.

Belanche, A., M. Doreau, J.E. Edwards, J.M. Moorby, E. Pinloche, and C.J. Newbold. 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. J. Nutr. 142:1684-1692.

Copelin, J.E., J.L. Firkins, M.T. Socha, and C. Lee. 2021. Effects of diet fermentability and supplementation of 2-hydroxy-4-(methylthio)-butanoic acid and isoacids on milk fat depression: 1. Production, milk fatty acid profile, and nutrient digestibility. J. Dairy Sci. 104:1591-1603.

de Souza, J., C.M. Prom, and A.L. Lock. 2021. Altering the ratio of dietary palmitic and oleic acids affects nutrient digestibility, metabolism, and energy balance during the immediate postpartum in dairy cows. J. Dairy Sci. 104:2910-2923.

Dehority, B.A., H.W. Scott, and P. Kowaluk. 1967. Volatile fatty acid requirements of cellulolytic rumen bacteria. J. Bacteriol. 94:537-543.

dos Santos Neto, J.M., J. de Souza, and A.L. Lock. 2021. Nutrient digetibility and production responses of lactating dairy cows when saturated free fatty acid supplements are included in diets: A meta-analysis. J. Dairy Sci. 104:12628-12646.

Ferraretto, L.F., P.M. Crump, and R.D. Shaver. 2013. Effect of cereal grain type and corn grain harvesting and processing methods on intake, digestion, and milk production by dairy cows through a meta-analysis. J. Dairy Sci. 96:533-550.

Firkins, J.L. 2021. Invited Review: Advances in rumen efficiency. Appl. Anim. Sci. 37:388-403.

Firkins, J.L., A.N. Hristov, M.B. Hall, G.A. Varga, and N.R. St-Pierre. 2006. Integration of ruminal metabolism in dairy cattle. J. Dairy Sci. 89(E. Suppl.):E31-E51.

Gorosito, A.R., J.B. Russell, and P.J. Van Soest. 1985. Effect of carbon-4 and carbon-5 volatile fatty acids on digestion of plant cell wall in vitro. J. Dairy Sci. 68:840-847.

Hackmann, T.J. and J.L. Firkins. 2015. Maximizing efficiency of rumen microbial protein production. Front. Microbiol. 6:465.

Hultquist, K.M. and D.P. Casper. 2016. Effects of feeding rumen-degradable valine on milk production in late-lactating dairy cows. J. Dairy Sci. 99:1201-1215.

Jackson, D.R., C.D. Cassilly, D.R. Plichta, H. Vlamakis, H. Liu, S.B. Melville, R.J. Xavier, and J. Clardy. 2021. Plasmalogen biosynthesis by anaerobic bacteria: Identification of a two-gene operon responsible for plasmalogen production in Clostridium perfringens. ACS Chemical Biology 16:6-13.

Jenkins, T.C. 1993. Lipid metabolism in the rumen. J. Dairy Sci 76:3851-3863.

Kaiser, J.C. and D.E. Heinrichs. 2018. Branching out: Alterations in bacterial physiology and virulence due to branched-chain amino acid deprivation. mBio 9:e01188-01118.

Kajikawa, H., M. Mitsumori, and S. Ohmomo. 2002. Stimulatory and inhibitory effects of protein amino acids on growth rate and efficiency of mixed ruminal bacteria. J. Dairy Sci. 85:2015-2022.

Kajikawa, H., M. Mitsumori, K. Tajima, and M. Kurihara. 2005. Short communication: Amino acids antagonistic to the amino acids inhibitory for growth rate of mixed ruminal bacteria. J. Dairy Sci. 88:2601-2603.

Kelly, W.J., S.C. Leahy, J. Kamke, P. Soni, S. Koike, R. Mackie, R. Seshadri, G.M. Cook, S.E. Morales, C. Greening, and G.T. Attwood. 2019. Occurrence and expression of genes encoding methyl-compound production in rumen bacteria. Anim. Microbiome 1:15.

Koike, S. and Y. Kobayashi. 2009. Fibrolytic rumen bacteria: Their ecology and functions. Asian-Australas. J. Anim. Sci. 22:131-138.

Lee, C., J.E. Copelin, T. Park, K.E. Mitchell, J.L. Firkins, M.T. Socha, and D. Luchini. 2021. Effects of diet fermentability and supplementation of 2-hydroxy-4-(methylthio)-butanoic acid and isoacids on milk fat depression: 2. Ruminal fermentation, fatty acid, and bacterial community structure. J. Dairy Sci. 104:1604-1619.

Liu, C., C. Wang, W.Z. Yang, B. Zhang, X.M. Yang, D.C. He, P. Zhang, K.H. Dong, and Y.X. Huang. 2009. Effects of isobutyrate on rumen fermentation, lactation performance and plasma characteristics in dairy cows. Anim. Feed Sci. Technol. 154:58-67.

Liu, Q., C. Wang, G. Guo, W.J. Huo, S.L. Zhang, C.X. Pei, Y.L. Zhang, and H. Wang. 2018. Effects of branched-chain volatile fatty acids on lactation performance and mRNA expression of genes related to fatty acid synthesis in mammary gland of dairy cows. Animal 12:2071-2079.

Moon, M. and K.L. Anderson. 2001. Eubacterium cellulosolvens alters its membrane protein, lipoprotein, and fatty acid composition in response to growth on cellulose. Anaerobe 7:227-236.

Moraïs, S. and I. Mizrahi. 2019. Islands in the stream: From individual to communal fiber degradation in the rumen ecosystem. FEMS Microbiol. Rev. 43:362-379.

Mukhopadhya, I., S. Moraïs, J. Laverde-Gomez, P.O. Sheridan, A.W. Walker, W. Kelly, A.V. Klieve, D. Ouwerkerk, S.H. Duncan, P. Louis, N. Koropatkin, D. Cockburn, R. Kibler, P.J. Cooper, C. Sandoval, E. Crost, N. Juge, E. A. Bayer, and H.J. Flint. 2018. Sporulation capability and amylosome conservation among diverse human colonic and rumen isolates of the keystone starch-degrader Ruminococcus bromii. Environ. Microbiol. 20:324-336.

Naas, A.E. and P.B. Pope. 2020. Ruminal fibre digestion. Pages 349-381 in Improving Rumen Function. C. Mcsweeney and R.I. Mackie, ed. Burleigh Dodds Science Publishing, Sawston, Cambridge, U.K.

National Academies of Sciences, E. and Medicine. 2021. Nutrient Requirements of Dairy Cattle: Eighth Revised Edition. The National Academies Press, Washington, DC.

Oba, M. 2011. Review: Effects of feeding sugars on productivity of lactating dairy cows. Can. J. Anim. Sci. 91:37-46.

Oba, M. and M.S. Allen. 1999. Evaluation of the importance of the digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. J. Dairy Sci 82:589-596.

Papas, A.M., S.R. Ames, R.M. Cook, C.J. Sniffen, C.E. Polan, and L. Chase. 1984. Production responses of dairy cows fed diets supplemented with ammonium salts of iso C-4 and C-5 acids. J. Dairy Sci. 67:276-293.

Patton, R.A., A.N. Hristov, and H. Lapierre. 2014. Protein feeding and balancing for amino acids in lactating dairy cattle. Vet. Clin. N. Am.-Food A. 30:599-621.

Peirce-Sandner, S.B., A.M. Papas, J.A. Rogers, T.F. Sweeney, K.A. Cummins, H.R. Conrad, and L.D. Muller. 1985. Supplementation of dairy cow diets with ammonium salts of volatile fatty acids. J. Dairy Sci. 68:2895-2907.

Roman-Garcia, Y., B.L. Denton, K.E. Mitchell, C. Lee, M.T. Socha, and J.L. Firkins. 2021a. Conditions stimulating NDF degradation by dosing branched chain volatile fatty acids. I: Comparison with branched chain amino acids and forage source in ruminal batch cultures. J. Dairy Sci. 104:6739-6755.

Roman-Garcia, Y., K.E. Mitchell, C. Lee, M.T. Socha, T. Park, B.A. Wenner, and J.L. Firkins. 2021b. Conditions stimulating NDF degradation by dosing branched chain volatile fatty acids. III: Relation with solid passage rate and pH on prokaryotic fatty acid profile and community in continuous culture. J. Dairy Sci. 104:9868-9885.

Roman-Garcia, Y., R.R. White, and J.L. Firkins. 2016. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. I. Derivation of equations. J. Dairy Sci. 99:7918-7931.

Russell, J.B., R.E. Muck, and P.J. Weimer. 2009. Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen. FEMS Microbiol. Ecol. 67:183-197.

Saluzzi, L., A. Smith, and C.S. Stewart. 1993. Analysis of bacterial phospholipid markers and plant monosaccharides during forage degradation by Ruminococcus flavefaciens and Fibrobacter succinogenes in co-culture. J. Gen. Microbiol. 139:2865-2873.

Sok, M., D.R. Ouellet, J.L. Firkins, D. Pellerin, and H. Lapierre. 2017. Amino acid composition of rumen bacteria and protozoa in cattle. J. Dairy Sci. 100:5241-5249.

Tebbe, A.W., M.J. Faulkner, and W.P. Weiss. 2017. Effect of partitioning the nonfiber carbohydrate fraction and neutral detergent fiber method on digestibility of carbohydrates by dairy cows. J. Dairy Sci. 100:6218-6228.

Terry, S.A., A. Badhan, Y. Wang, A.V. Chaves, and T.A. McAllister. 2019. Fibre digestion by rumen microbiota — A review of recent metagenomic and metatranscriptomic studies. Can. J. Anim. Sci. 99:678-692.

Trautman, A., L. Schleicher, S. Deusch, J. Gätgens, J. Steuber, and J. Seifert. 2020. Short-chain fatty acids modulate metabolic pathways and membrane lipids in Prevotella bryantii B14. Proteomes 8:28

Wang, C., Q. Liu, G. Guo, W.J. Huo, Y.L. Zhang, C.X. Pei, and S.L. Zhang. 2019. Effects of rumen-protected folic acid and branched-chain volatile fatty acids supplementation on lactation performance, ruminal fermentation, nutrient digestion and blood metabolites in dairy cows. Anim. Feed Sci. Technol. 247:157-165.

Weld, K.A. and L.E. Armentano. 2017. The effects of adding fat to diets of lactating dairy cows on total-tract neutral detergent fiber digestibility: A meta-analysis. J. Dairy Sci. 100:1766-1779.

White, R.R., Y. Roman-Garcia, and J.L. Firkins. 2016. Meta-analysis of postruminal microbial nitrogen flows in dairy cattle. II. Approaches to and implications of more mechanistic prediction. J. Dairy Sci. 99:7932-7944.

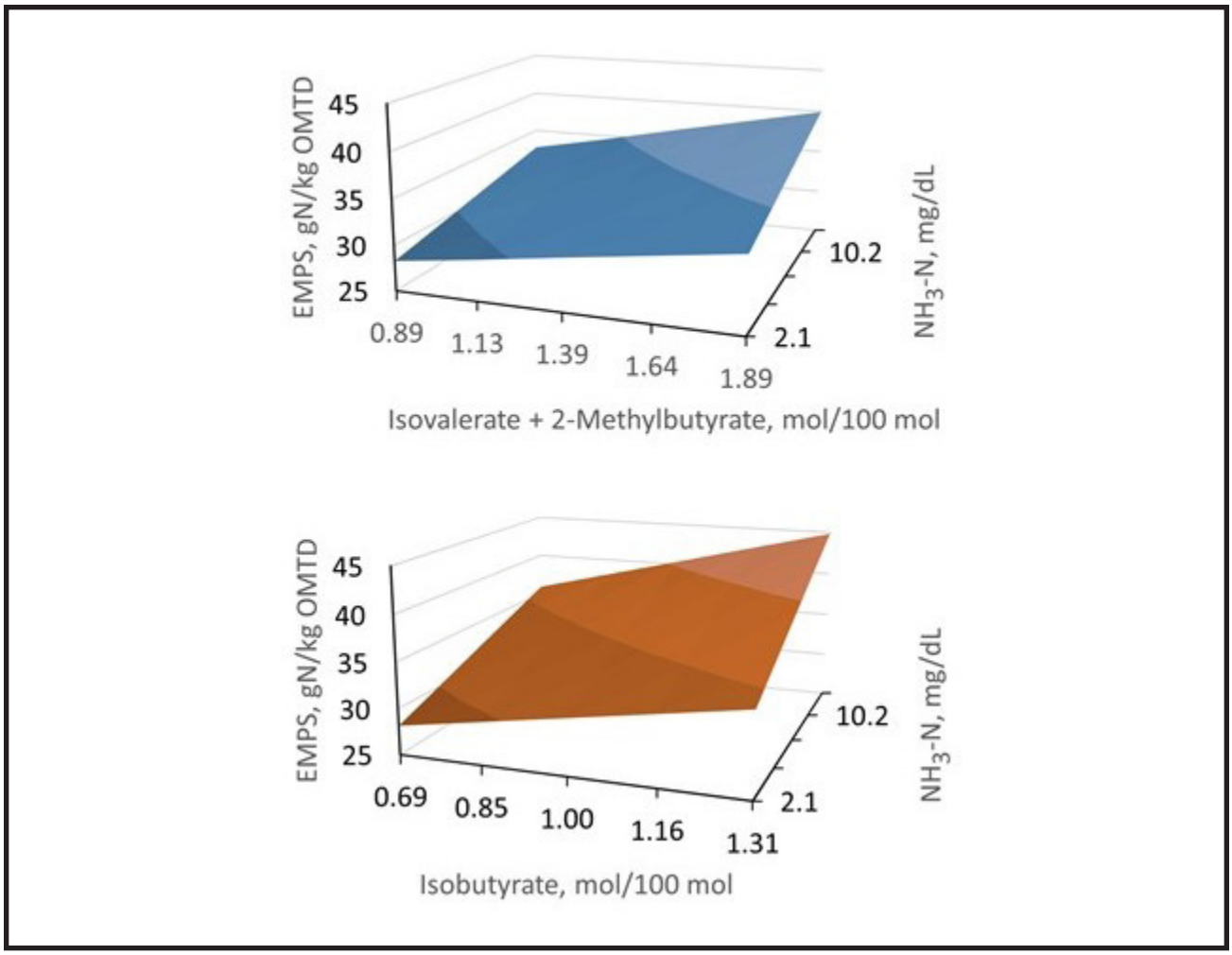

Figure 1. Predicted efficiency of microbial protein synthesis (EMPS, grams microbial N/kg of organic matter truly degraded in the rumen) is predicted to increase from the combination of Ival and 2MB in their common coelution or from Ibu) and with increasing ammonia-N concentration in dairy cattle (Roman-Garcia et al., 2016).

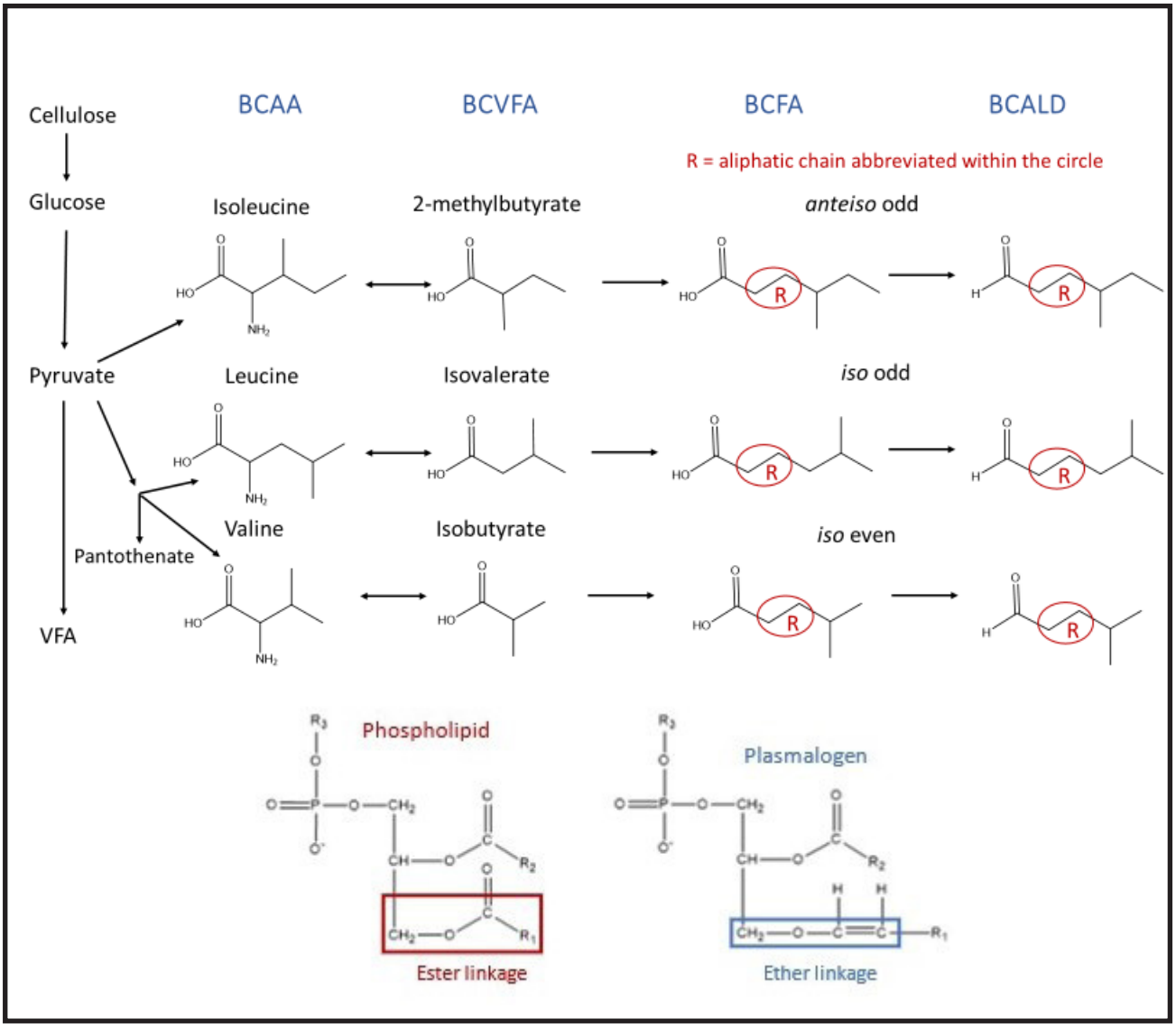

Figure 2. Branched chain amino acids (BCAA) can be produced from pyruvate and then converted to branched chain volatile fatty acids (BCVFA) for conversion to branched chain fatty acids (BCFA) or branched chain aldehydes (BCALD). Many of the cellulolytic bacteria cannot produce BCAA and therefore rely on exogenous BCVFA for both formation of BCAA or to BCFA and BCALD. In the bottom figure, the BCALD (or straight chain fatty aldehydes) are shown connected to the first carbon of glycerol by an ether linkage that allows a vinyl (double) bond in plasmalogens. The BCFA (or straight chains such as palmitic acid) are connected by ester bonds either to the second carbon of plasmalogens or to the first or second carbon in phospholipids.