Reconsidering rumen microbial consortia to enhance feed efficiency and reduce environmental impact of ruminant livestock production systems

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ABSTRACT - Because cultivation-based approaches to assess rumen microbiology assess only the minority of microbial groups and can miss opportunities to assess important functions, molecular-based approaches have flourished in microbial ecology and have been adapted to the rumen. Current approaches are described herein, specifically for a robust adaptation to nutrition studies or future opportunities. These included automated profiling techniques, density gradient gel electrophoresis, and future adaption of microarray or high-throughput sequencing technologies. Based on current knowledge, a more holistic approach is needed to describe various functional groups of microbes within the context of how they influence, and are influenced by, the whole consortium (combination of microbial groups). Such a perspective is applied to issues related to increasing fiber digestibility when feeding concentrate or unsaturated fats to high producing beef and dairy cattle. These same microbial populations should help to provide growth factors for fibrolytic bacteria while competing against the hyperammonia-producing bacteria such that there would be less need for excessive rumen-degraded protein as a safety factor. Moreover, these same dietary conditions influence the processes of biohydrogenation and methanogenesis. After accounting for population structures of bacteria, protozoa, methanogenic archaea, and even fungi, efforts to integrate molecular-based rumen microbial ecology with dietary conditions should allow us to better explain and therefore predict conditions that will improve feed efficiency and reduce environmental impact of ruminant production systems.

Key Words: biohydrogenation, methanogenesis, rumen fiber digestibility, rumen microbial ecology

Consórcios microbianos no rúmen para melhorar a eficiência alimentar e reduzir o impacto ambiental dos sistemas de produção animal

RESUMO - Técnicas tradicionais de identificação de microorganismos ruminais utilizando metodologias de cultivo conseguem identificar um pequeno grupo de bactérias. Técnicas de identificação molecular têm sido amplamente utilizadas em ecossistemas microbiológicos e adaptadas em estudos com ruminantes. Fundamentado no conhecimento atual, uma abordagem mais holística é necessária para descrever vários grupos funcionais de microorganismos dentro do contexto de como eles influenciam e são influenciados dentro do ecossistema ruminal (associação de grupos de microrganismos). Essa perspectiva é aplicada a questões relacionadas com a digestibilidade da fibra, quando o nível de concentrado ou gorduras insaturadas é elevado na dieta, para bovinos de altas produções de carne e leite. Essas mesmas populações microbianas devem ajudar a fornecer fatores de crescimento para as bactérias fibrolíticas enquanto competem com as bactérias hiperprodutoras de amônia de tal forma que haveria menor necessidade de degradação excessiva de proteínas no rúmen como um fator de segurança. Além disso, estas mesmas condições alimentares influenciam os processos de biohidrogenação e metanogênese. Depois de contabilizar as estruturas da população de bactérias, protozoários, bactérias metanogênicas e até mesmo fungos, os esforços para integrar a base molecular da microbiota ruminal com as condições alimentares deverá permitir-nos explicar melhor e, portanto, prever as condições que irão melhorar a eficiência alimentar e reduzir o impacto ambiental dos ruminantes em sistemas de produção.

Palavras-chave: biohidrogenação, digestibilidade da fibra no rúmen, ecologia microbiana ruminal, metanogênese

Introduction

As explained more thoroughly in previous reviews (Firkins et al., 2007, 2008a), approaches to improve efficiency of ruminant production must be meshed with societal issues to reduce the impact of that production on air and water quality. Empirical meta analyses from a compilation of published articles and improved computer modeling can further improve the efficiency of conversion of feed nutrients into animal products. For example, we need to explain better why increasing supply of rumen-degraded starch depresses NDF digestibility in mixed diets (Firkins et al., 2001; Nousiainen et al., 2009). However, these results account for a large amount of among-

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experiment variation that remains to be explained and are based on predicting an average animal's needs over the 24-h interval of the average day. In contrast, we need to account further for among-animal variability in ruminal pH, digestibility of protein and fiber, accumulation of bioactive intermediates from fatty acid biohydrogenation, and methane production to manipulate diets to improve feed efficiency and reduce environmental impact.

After the advent of systematic molecular-based techniques, rumen microbiologists have continued to expand the boundaries of our knowledge relating microbial ecology to ruminant nutrition. The proportion of rumen microbial phylotypes that are represented by cultured isolates is low (Edwards, 2008), but we need to move beyond "the standard conclusion that microbial ecosystems are remarkably complex and diverse" that limits microbial ecological approaches (Tringe & Hugenholtz, 2008). Future approaches will increase the depth of coverage of the diversity of the rumen microbiome but likely will continue to be constrained to a relatively few number of samples until sequencing throughput and computing power increases systematic analysis and our interpretation of those extensive data. Until then, more rapid (but less comprehensive) approaches can survey general shifts in microbial structure in nutrition studies but also can narrow down which specific samples would be more beneficial for the in-depth approaches. The objectives of this paper are to describe and put forward potential opportunities for enhanced application of microbial ecology techniques to explain why mixed forage and concentrate reduces fiber digestibility in the rumen, how microbial populations influence ruminal biohydrogenation of unsaturated fat, and opportunities to reduce emission of methane and excretion of nitrogen in urine.

Mining the tibosome to enhance our knowledge of the rumen microbiome

In the past 10-15 years, there has been considerable progress in our understanding of the complexity of the rumen microbiome. Because of the critical and universal need for ribosomal protein synthesis for microbial growth, rRNA was identified as a foundational tool to evaluate bacterial taxonomy (Firkins & Yu, 2006), particularly those that have not been represented by strains that have been cultured and characterized phenotypically (e.g., enzyme activities or fermentation end-products). The 16S rRNA subunit was originally recruited because of its intermediate size to capture an optimum diversity among species while still not being too big to take enormous amounts of time by early sequencing methods. Thus, 16S rRNA databases

have large and diverse amounts of sequence information available and have been the basis for dozens or hundreds of studies in microbial ecology. Its combination of highly conserved regions flanking several hypervariable regions has allowed researchers to design primers in polymerase chain reaction (PCR) to compare the diversity of microbial populations, including those that have not (yet) been cultured. Because PCR starts losing efficiency after several hundred base pairs, the smaller hypervariable regions can be adapted for use in less expensive and more rapid approaches that profile abundant populations (Mackie et al., 2007). By using primers that span two subunits of the rRNA (the intergenic spacer region), the resulting sequences might capture more diversity; however, because there currently are fewer sequences covering this region in the databases, the resulting sequence analysis can be more challenging to relate to specific groups of bacteria. Firkins and Yu already have described in detail the foundational studies mostly based on dendrograms of sequences from the entire 16S rRNA gene (rDNA) that had been inserted into clone libraries, and these will not be further described because of brevity but also because newer techniques offer either deeper coverage or more systematic adaptation to nutrition studies. Also, 16S rDNA sequence analysis for phylogenetic characterization does not necessarily explain diverse niches among closely related groups (Firkins et al., 2008a).

The rumen bacterial community, previously suggested to have 300 to 400 species (Edwards, 2008), which typically are classified as operational taxonomic units with 97% rDNA sequence similarity, probably has 2000 or more species based on preliminary data from current microarray efforts at Ohio State. More extensive analyses for the human gut suggests about 5000 species (Zoetendal et al., 2009), and those authors implored the need to further characterize the roles of many phylotypes that are characterized only by sequence information, including efforts to isolate pure cultures or more extensive metagenomics approaches. Even so, there is considerable redundancy among different populations occupying the same niche (Edwards, 2008), so any true approximation of microbial function requires thousands, not hundreds or fewer (as in most studies) sequences (Firkins & Yu, 2006; Youssef & Elshahed, 2008). Finally, any serious attempt to relate fiber digestibility to microbial structure needs to extract DNA or RNA from the particulate fractions of rumen contents, whereas too many prior studies have extracted bacteria only from the fluid phase (Firkins & Yu, 2006; Flint et al., 2008), and even detached particulate-phase bacteria might differ from the

native attached bacteria (Ramos et al., 2009). Although there clearly are adherent bacteria such as uncharacterized clostridia that contribute to fiber degradation (Larue et al., 2005), characterized cellulolytic fibrobacters and ruminococci still are thought to have a major role in this niche (Wallace, 2008). Consequently, molecular approaches targeting these species are being used to evaluate the relative abundance (Denman & McSweeney, 2006) and location (Shinkai & Kobayashi, 2007) of these species as they colonize forages particles.

Molecular approaches to integrate microbial ecology with ruminant nutrition

There are numerous approaches that have been used in molecular-based profiling of bacterial communities (Li et al., 2009), and these emerging technologies are becoming far more feasible for ruminant nutrition studies. Microarray and high-throughput sequencing allow a far more robust and deeper characterization and can reduce the time, expense, and potential biases from preparatory PCR or cloning approaches (Zoetendal et al., 2009;Smith & Osborn, 2009). Because those approaches are limited in current adaptation to a variety of samples, though, other automated approaches have been adapted for studies evaluating important problems in ruminant nutrition (Khafipour et al., 2009;Welkie et al., 2010). Automated ribosomal intergenic spacer analysis has limitations and benefits (Popa et al., 2009). For example, signals are unique and reproducible, but some signals might not identify a unique strain or species. Also, the major populations that caused those shifts can be difficult to identify without quantitative PCR verification, which could be limited by primer design and verification. A similar limitation has been documented for amplified ribosomal DNA restriction analysis (Sklarz et al., 2009). However, these techniques still offer rapid coverage of numerous abundant species (or potentially more than one species that have the same signal), and the results can be inputted into computer programs that can relate shifts in those signals to diet or animal responses. For a semiquantitative compromise, users can consider serially diluting samples prior to automated bacterial profiling (Ramette, 2009).

Gel-based density gradient gel electrophoresis (DGGE) has been used widely for systematic analyses of population shifts among rumen microbial populations. There might be fewer groups assessed (although newer technology can increase its comprehensiveness), but an advantage is that bands can be cut and sequenced for inferences about specific populations associated with shifts in banding profile (Karnati et al., 2009). Although there have been some

issues raised about specificity, exclusivity, and the ability of a smaller sequence to represent the whole 16S rRNA gene, these issues can be overcome, at least enough so that DGGE results can be integrated with nutrition studies (Guan et al., 2008;Firkins et al., 2008a). Useful examples of DGGE include shifts in the total bacterial and butyrivibrio communities (Kim et al., 2008), verification of collection site for bacteria (Lodge-Ivey et al., 2009), bacterial detachment procedures (Martínez et al., 2009), shifts in protozoal populations (Boeckaert et al., 2007), a shift in bacterial populations resulting from defaunation (Karnati et al., 2009), and verification that protozoal samples collected from the rumen actually represent the protozoa passing to the duodenum (Sylvester et al., 2005;Yáñez-Ruiz et al., 2006).

Forms of principal components analyses have been used to statistically associate microbial populations to increased incidence of subacute rumen acidosis (Khafipour et al., 2009) and milk fat depression (Weimer et al., 2010). These approaches certainly enhance objectivity and comprehensiveness compared with user-based explanations of population shifts, but they still need some caveats. A recent analysis of a non-rumen microbiome has documented an improvement to the standard principal components analysis for DGGE (Illian et al., 2009). Readers need to be aware that condensing common variance to a few principal components should improve the explanation of complex relationships or else it really makes little advance in our knowledge. For example, if there are more than three components or if two are nearly of equal weight, this analysis could be biased or limited in benefit. Also, components should not necessarily be limited to linear relationships (most biological response functions are nonlinear), and sometimes the variables (typically measured experimentally) might actually be more useful for explaining relationships than if those variables are embedded into some more nebulous component that is not directly measured.

Whether to verify microarray results or for a priori hypotheses for changes in targeted bacterial species or groups, real-time quantitative PCR (qPCR) has become the standard for quantitative analyses of microbial population abundances. We described a number of potential biases, including DNA extraction recovery, variable gene copy number per cell, PCR amplification issues, the need to derive a DNA standard for each sample, and the number of analyses needed to overcome the low precision (small errors are exponentially amplified with multiple cycles); we also provided spiking approaches to account for losses in

recovery and the potential for the background in the sample to affect qPCR (Sylvester et al., 2004). To avoid many of these analyses, some researchers have chosen to use a relative quantification by normalizing the 16S rDNA (or other gene) copies from specific primers relative to the total bacterial rDNA copies using universal primers (for example, see McSweeney & Denman, 2007) . Another approach has been to use a serial dilution series of the targeted species relative to a dilution of the total bacterial rDNA (Weimer et al., 2008). These are clearly improvements with respect to traditional approaches to simply report the uncorrected rDNA copies, but even relative (normalized) qPCR can be biased by shifts within the bacterial community composition (Smith & Osborn, 2009).

The 16S rDNA copies can vary between 1 and 15 per cell between species of bacteria (Smith & Osborn, 2009). Clearly, bacteria will increase in size in proportion to increases in growth rate, but this is largely a reflection of the pre-division need to double the genome prior to cell division (Haeusser & Levin, 2010). The rDNA copies are in the thousands and can fluctuate considerably per protozoan cell (Sylvester et al., 2009). Because the eukaryotic cell cycle is more complex than that of bacteria, proper collection of representative protozoal samples is critical (Sylvester et al., 2005), and RNA concentration presumably would vary many fold more.

Considerable advancement has been made with respect to increasing the yield and reproducibility of RNA extraction for subsequent microbial profiling. Interfering compounds, RNAses, and other background effects vary RNA stability among complex microbial ecosystems (Nocker & Camper, 2009). Recent improvements in recovery and reproducibility of RNA extraction from rumen samples (Béra-Maillet et al., 2009; Kang et al., 2009) offer considerable promise. The concentration of RNA increases within cells after adding substrate (Lu et al., 2009), or, conversely, substrate deprivation should reduce the concentration of RNA per cell (Russell, 2007). Also, the greater amount of RNA, compared with DNA, per cell might reduce amplification biases that are needed to increase the yield of DNA enough for microbial profiling (Hoshino et al., 2007). After reverse transcriptase, the resulting cDNA libraries might be more representative of the community structure after that dietary change (Kang et al., 2009). Moreover, reverse transcriptase-qPCR has the potential to improve the quantitative evaluation of such population shifts using 16S rRNA (Smith & Osborn, 2009) or could be related more functionally to mRNA distributions (Jahn et al., 2008).

Microbial consortia efficiently degrade fiber in the rumen

There is a core population of particle-associated bacteria that appears to be very resilient (resistant to dietary changes), whereas the structure of the fluid-associated bacteria is far more variable and dynamic (Wallace, 2008;Welkie et al., 2010). However, these groups have important interactions with each other. Colonization is extensively committed within 5 to 15 min and thereafter is a function of subsequent biofilm formation (Edwards et al., 2007). Bacterial enzymes attacking plant particles were characterized as initially arising from generalist bacteria followed subsequently by enzymes for the more recalcitrant polymers (Brulc et al., 2009). Incubating forage preparations in vitro documented large differences among *Fibrobacter succinogenes* isolates at 15 min of adhesion without correlation to digestibility (Shinkai et al., 2009), whereas subsequent work documented a progressive colonization that depended on the strain of *F. succinogenes* initiating colonization prior to inoculation with ruminal fluid (Shinkai et al., 2010). Because of the complexity of enzymes involved in degradation of polymeric cellulose (Flint et al., 2008), such a need for a close physical environment would seem necessary for all highly fibrolytic bacteria, including *Ruminococcus flavefaciens*, which encodes genes for a complex and adept ability to bind to cellulose (Berg Miller et al., 2009). Substrate surface area likely limits degradative capacity and subsequent growth of the colonizing bacteria, which then further increases enzyme capacity to degrade those particles rapidly before they pass out of the rumen. Thus, the first hour of colonization might dictate the efficiency of fiber degradation of feed during its entire residence time in the rumen (Mouriño et al., 2001).

Some redundancy should add resilience to a system, and Edwards et al. (2000) explained why a more holistic approach is needed to understand the temporal and spatial dynamics of microbial degradation in the rumen. Certain strains of *F. succinogenes* and fungi are more adept at degrading poor quality grasses (Denman & McSweeney, 2006;McSweeney & Denman, 2007). Different cellulolytic strains probably have different niches according to the varying recalcitrance of plant fiber (Shinkai & Kobayashi, 2007; Shinkai et al., 2009;Shinkai et al., 2010), and they probably even differentially express enzymes contributing to overall cellulolysis (Béra-Maillet et al., 2009). Fiber sources are typically degraded at about 5%/hour, gradually releasing sugars and oligosaccharides from both cellulose (Wells et al., 1995) and hemicellulose (Cotta & Forster, 2006) that are used by the entire bacterial community. In return, these secondary bacteria degrade protein and provide growth

factors for the primary cellulolytics. This model can be supported by studies in which enzymes were exogenously applied to feed (Beauchemin et al., 2003). Even addition of exogenous amylase (Kingerman et al., 2009) or protease (Colombatto & Beauchemin, 2009) with insignificant activities against fiber still stimulated NDF digestibility. The authors referenced a proposed mechanism of increased surface area and stimulation of non-cellulolytics to cross-feed with cellulolytic bacteria. However, we need to look beyond the classical cross-feeding dogma. Even strains of *Prevotella* species, typically considered for its amylolytic and proteolytic capacity, can contribute significantly to hemicellulose degradation, perhaps through the ability to hydrolyze ferulic acid esters (Dodd et al., 2009). Non-core populations could degrade tannins or other compounds impeding fibrolytic colonization, and there could be a physical benefit to the non-motile cellulolytic populations by highly motile spirochaete (corkscrew-shaped) bacteria (Shinkai et al., 2010).

The primary cellulolytic bacteria, which also contribute to hemicellulose degradation, are intolerant to a low ruminal pH. Clearly, too much grain in the diet will reduce fiber digestibility, especially the non-forage NDF that has a high potential digestibility and faster passage rate than forage NDF (Firkins, 1997). This decreased fiber digestibility, especially of forages, is important because it could promote bulk fill limitation of voluntary feed intake. Low ruminal pH could be reducing binding by cellulolytics to particulate matter in the rumen, allowing acid-tolerant bacteria to initially adhere and therefore have a more favorable competition for new feed particles (Mouriño et al., 2001). However, although the latter reference (and much of our thinking) focuses on ruminal pH < 6.0 as the main limitation of fiber digestibility, more current work with molecular techniques has shown that even cows with very low pH can maintain normal populations of cellulolytic bacteria (Palmonari et al., 2010). Even grain-induced acidosis did not reduce the abundance of cellulolytic bacteria unless it progressed to what was classified as severe acidosis (Khafipour et al., 2009). Russell et al. (2009) discussed how low pH per se did not increase maintenance energy costs of bacteria. Calsamiglia et al. (2008) varied forage:concentrate while maintaining different constant pH values in continuous culture. Adding acid to systematically decrease pH had a direct effect that was dose-responsive and predictive for many variables, and was much more critical than forage:concentrate. However, while confirming the important general inhibition by low pH, these pH values were maintained constantly and were distributed throughout the entire flasks by rapid stirring of pH-controlled buffer

with finely ground feed particles. In contrast, ruminal pH depends on the time after feeding and on digestibility of starch, not just buffering capacity (Lechartier & Peyraud, 2010).

In studies investigating the "carbohydrate effect" independent of pH (Piwonka & Firkins, 1996), we discussed the potential for higher glucose supply to antagonize fiber degradation through production of proteinaceous inhibitors. These "alternatives to antibiotics" are ubiquitously produced, especially by gram-positive bacteria (Russell & Mantovani, 2002), but in vivo impact is not clear. One such protein inhibitor is "bovicin" produced by *Streptococcus bovis*, which probably is the bacterium most attributed to promoting ruminal acidosis. Interestingly, this compound was shown to be quite inhibitory to one representative of a low abundance/high activity group termed "hyperammonia-producing bacteria" (HAB) (Mantovani & Russell, 2002). Although secreted bovicin can be rapidly degraded by proteases and peptidases in the rumen, the membrane-associated bovicin could resist degradation and still have significant activity (Lima et al., 2009). Similarly, some strains of butyrivibrios produce bacteriocins that strongly inhibit HAB (Walker et al., 2005).

Those HAB that have been cultivated are inhibited by low pH, but a computer model suggested a more direct antagonism by carbohydrate-fermenting bacteria against the HAB population (Rychlik & Russell, 2000). Besides the direct antagonism via proteinaceous inhibitors that they suggested, though, the low abundance HAB compete for the same amino acid pool with the far more abundant saccharolytic bacteria. Unlike other amylolytic bacteria that have a moderate rate of deamination and that use the carbon skeletons for only a portion of their energy, the HAB are obligate amino acid fermenters that rapidly reduce the availability of amino acids to stimulate growth of amylolytic, hemicellulolytic, or cellulolytic bacteria. *S. bovis* will increase its growth rate by reducing energy spilling when it has adequate access to preformed amino acids (Russell, 2007), so this sugar user and probably others could benefit a balanced microbial consortium by inhibiting or outcompeting the HAB. Thus, moderate provision of grain could be limiting these obligate deaminators and maintaining a more consistent concentration of peptides and amino acids between meals to maintain a more efficient microbial ecosystem involved in fiber degradation.

Various additives are being explored for their inhibition against the HAB (Patra & Saxena, 2009), and more work is needed (Calsamiglia et al., 2007). However, future researchers need to consider that various microbial populations can adapt to monensin (Lima et al., 2009), tannins (Smith et al.,

2005), and essential oils (Patra & Saxena, 2009) through changing their membrane architecture or production of proteinaceous cell envelopes that impede the membrane-associated inhibition. Consequently, more in vivo experiments are needed to evaluate efficacy after adaption to these compounds.

Microbial fermentation and ruminal acid absorption

Acidosis risk among animals is not strongly related to rumination behavior (DeVries et al., 2009), and the rumen digestibility of carbohydrate is more important than forage NDF (Lechartier & Peyraud, 2010). Although chewing behavior is clearly related to ruminal pH over a broad range, within a narrow range of effective fiber concentration, susceptibility to acidosis bouts within a day probably depends more on VFA production and clearance from the rumen. Many, but not all, studies with sugars show increases in the molar proportion of butyrate or valerate (Heldt et al., 1999), and a recent molecular analysis of rumen contents from feedlot beef steers (not fed sugars) documented that those with improved feed efficiency had increased butyrate and valerate concentrations (Guan et al., 2008). Butyrate and valerate are much more important fuel sources for the rumen epithelium than are acetate or propionate (Kristensen, 2005). Butyrate is much more lipophilic than acetate and has a much faster passive absorption rate that is further accentuated by the rapid catabolism to β-hydroxybutyrate inside the cell; however, an increased absorption rate of butyrate compared with acetate was not realized, probably as a result of compensatory absorption by non-passive mechanisms (Aschenbach et al., 2009). For sheep fed the same acidosis challenge diets, those grouped as responders had increased rates of VFA absorption in vitro with correspondingly increased β-hydroxybutyrate concentrations in the blood compared with the non-responders that maintained a lower ruminal pH (Penner et al., 2009a). In addition to passive absorption removing a proton from the rumen, acetate can be absorbed as an anion with a corresponding exchange with a bicarbonate anion that would help buffer pH. Potential for transcriptional control of acetate transport proteins (Aschenbach et al., 2009) needs futher clarification with respect to dietary control of mRNA expression in dairy cattle (Penner et al., 2009b). Besides serving as fuel, butyrate probably controls gene expression and growth of rumen epithelial cells but likely depends on other dietary factors, considering that butyrate's control of gene expression and cell division of colonocytes has been extensively researched but interacts with diet, especially source of dietary fat (Lupton, 2004). Regardless of mechanism, these results

still document a greater variability among lactating cows fed the same diet and the potential for the need to maintain conditions that resist a decline in pH and lost microbial efficiency for some cows.

Lactic acid is well known to be a much stronger acid than the volatile fatty acids (VFA). Certain acid-tolerant microbes can ferment glucose to lactate at least five times faster than they ferment glucose to VFA, thus getting more total energy even from the less energetically efficient pathway of lactate production (Nagaraja & Titgemeyer, 2007). As those authors discussed, although this potential burst of lactate production can result in acute acidosis, lactic acid concentration rarely increases because its catabolism rate compensates as more is lactate is produced until pH progressively declines below 5.5 to inhibit the lactate consumers. Therefore, if we stimulate the lactate consumers through basal sugar feeding, this should provide a ready population to "buffer" against bursts of lactate production rate exceeding its fermentation rate. In support, subacute acidosis was associated with reduced bacterial diversity (Khafipour et al., 2009). Moreover, supplementing sugars could actually increase pH if there is increased production of VFA that are 4 carbons or greater in chain length because these pathways would condense two moles of acid into one, thus partially compensating for the high ruminal digestibility of sugars. Piwonka & Firkins (1996) estimated that lactate was converted to propionate and butyrate in vitro in a ratio of about 1 to 2, which is expected from other studies documenting the metabolic conversion of lactate to butyrate or valerate (Marounek et al., 1989; Duncan et al., 2004).

Improving efficiency of conversion of rumen-degraded protein into microbial protein

The ability to consume lactate and stimulate fiber digestibility probably depends on having adequate rumen-degraded protein (RDP). Both low pH (Calsamiglia et al., 2008) and low nitrogen per se (Griswold et al., 2003) can decrease proteolysis by bacteria in continuous culture. Many bacteria and protozoa can ferment lactate (Nagaraja & Titgemeyer, 2007), but *Megasphaera elsdenii* is most attributed to filling this niche based on in vitro (Rychlik et al., 2002) and in vivo (Klieve et al., 2003) data. A comprehensive metagenomics study confirmed the importance of this bacterium to keep lactate concentrations low in dairy cattle fed diets promoting acidosis (Khafipour et al., 2009). Besides *M. elsdenii*, though, increased *Prevotella* spp were associated with maintaining an efficient bacterial population structure in that study. The authors discussed the potential for a *Prevotella* probiotic

to reduce the incidence of subacute acidosis. This broad phylum is well documented for degrading starch, sugar, and even hemicellulose but also its major capacity to degrade proteins (Walker et al., 2005). Their active protease capacity funnels short peptides and amino acids to other bacteria for uptake and use for protein synthesis or for catabolism. Some strains of *M. elsdenii* have rapid rates of deamination, but not necessarily high proteolytic activity, and they likely use these amino acids more for energy after glucose or lactate become limiting (Rychlik et al., 2002). However, as explained by those authors, this predominant lactate fermenter could be an important producer of branched chain VFA available for cellulolytic bacteria that require them, effectively competing against the HAB to support a more balanced consortium (see prior discussion). When beef cattle were dosed with pure starch or glucose, lactate only spiked after feeding glucose, and there was a corresponding prolonged butyrate concentration (Arroquy et al., 2004b) that would indicate stimulation of a butyrate-producing population. In a companion study (Arroquy et al., 2004a), lactate concentration again spiked when glucose was dosed at an equivalent concentration as starch, but lactate concentration decreased incrementally back to baseline with increasing supply of RDP. In a carbon recovery analysis, Hall & Weimer (2007) surmised a considerable amount of peptide usage apparently for VFA production with increasing sucrose addition to mixed cultures. Thus, having adequate RDP might be a prerequisite for allowing the sugar-fermenting/lactate-fermenting populations to "buffer" the ruminal fermentation from bursts of low pH.

Research with isotopically labeled amino acids supports their stimulation of fiber digestibility (Newbold, 1999). Cellulolytic bacteria had stimulated growth rate by preformed amino acids but only when using cellobiose (the disaccharide repeating unit of cellulose) for very rapid growth; thus, the benefit to fiber degradation was attributed to stimulating the synergistic "non-cellulolytic partners". Thus, stimulation of proteolytic bacteria, without stimulating hyperammonia producers, should stimulate ruminal fiber digestibility, whereas over-feeding grain could negate this benefit by reducing pH or availability of growth factors. For example, adding sugars sometimes has decreased branched chain VFA concentrations (Heldt et al., 1999; Oelker et al., 2009), which could be a result of increased usage, not production, so long as the concentration is above the minimum needed to optimize fiber degradation.

In addition to providing peptides for bacterial growth, RDP also is continually degraded to ammonia, the main N source for cellulolytic bacteria. In a study with alfalfa as the exclusive forage and therefore a high dietary RDP, using ^{15}N as a tracer, Hristov et al. (2005) determined that the ammonia production rate was decreased by over 25% when glucose was fed rather than an equivalent amount of starch or byproduct fiber. Thus, there was considerably less irreversible loss of ammonia (i.e., absorption or conversion to microbial N leaving the rumen) while there was a greater reliance on amino acids or peptides for incorporation into microbial protein flowing from the rumen. The linear decrease in ammonia concentration with increasing sucrose substitution for starch without an increase in microbial N production is consistent with these responses (Broderick et al., 2008). The net concentration of ruminal ammonia depends on its production from RDP and blood urea N relative to its incorporation into microbial protein. Thus, a net decrease in ammonia concentration can be a sign of more efficient microbial protein synthesis that could be limited by peptide supply, not necessarily ammonia. Improving models with responses such as these should help optimize the conversion of RDP into microbial protein with less need for extra RDP safety factors that promote excessive loss of N in the urine (Firkins et al., 2007).

Many modeling efforts have focused on the three well characterized cellulolytic isolates: *F. succinogenes*, *R. flavefaciens* and *R. albus*. All require branched chain VFA and ammonia as the principal nitrogen source. The ruminococci also have a clear and critical requirement for phenylalanine (from RDP) for conversion to growth factors needed for adhesion to cellulose but not hemicellulose (Reveneau et al., 2003). Because branched-chain VFA are less likely to be limiting for their growth in the rumen compared with ammonia, most models focus on providing adequate ammonia concentration for the cellulolytic populations and on peptide supply for the amylolytic bacteria. The foundational studies and even a more current modeling effort supporting this contention (Russell et al., 2009). However, they have focused on the use of pure cultures and have typically ignored that these cellulolytics work in a consortium and benefit at least indirectly from preformed amino acids (Walker et al., 2005) as well as ignoring that a more substantial amount of hemicellulose is degraded by bacteria that are stimulated by amino acids (Griswold et al., 2003). Future research efforts should evaluate interaction between the non-structural carbohydrate bacteria and the structural carbohydrate bacteria for computer models.

Ruminal biohydrogenation

Although we have long known that *B. fibrisolvens* biohydrogenates polyunsaturated fatty acids, much about

the process, kinetics, and impact on ruminant products still remains unclear (Jenkins et al., 2008). Both depressed pH and increasing concentrate proportion in diets are well known to potentially depress milk fat through increased outflow of *trans* biohydrogenation intermediates, sometimes sporadically among different cows fed the same diet and with shifts in bacterial populations (Weimer et al., 2010). To sort out some of these responses, continuous culture has been used to establish conditions to try to better document a mechanism. Continuously maintaining pH at 5.6 dramatically elevated the outflow of 18:1 *trans*-10, and quantitative PCR analysis of biohydrogenating bacterial groups suggested a change in the group that reduces 18:1 *trans* isomers to 18:0 (Fuentes et al., 2009). This result is in contrast with speculation that the low pH inhibited this final step in biohydrogenation (Qiu et al., 2004). Because pH was maintained at 6.2 in another continuous culture study, the linear decrease in biohydrogenation associated with increased sucrose addition was attributed to shifts in bacterial populations (Ribeiro et al., 2005).

Future efforts should separate the rates of lipolysis from the kinetics of biohydrogenation (Jenkins et al., 2008). *Anaerovibrio lipolytica* and related lipolytic bacteria are sensitive to low pH, but its abundance decreased more from subacute acidosis introduced by alfalfa pellets replacing long forage compared with an acidosis challenge from high concentrate (Khafipour et al., 2009). Certain bacteria encode genes for enzymes used in biohydrogenation to decrease inhibitory effects of unsaturated fatty acids. That a mechanism is likely related to uncoupling of ATP production in the presence of inhibitory levels of unsaturated fatty acids, a potential inhibition by lactate, and inhibition of the butyrate kinase pathway for ATP synthesis from butyrate formation deserves future attention (Maia et al., 2010).

Rumen protozoa probably also contribute to lipolysis but only indirectly influence the accumulation of *trans* fatty acids by incorporating unsaturated fatty acids or intermediates such as conjugated linoleic acids, thereby keeping them out of the biohydrogenation pool (Firkins et al., 2008a). Algal lipids can influence the protozoal population structure (Boeckaert et al., 2007). Fish oil (some of which could be from algal fatty acids accumulating through the food chain) also can influence the butyrivibrio population structure (Kim et al., 2008). Although those authors suggested that there was a linear relationship between the stearate-producing butyrivibrios and 18:0 flow to the duodenum, this relationship was weak and might have been abolished had they included treatment as a class variable in the model. They also extracted DNA from ruminal

fluid, whereas the particulate phase was suggested to harbor the final stearate-producing butyrivibrios (Boeckaert et al., 2009). Finally, protozoa probably influence biohydrogenation in an indirect way by influencing the population structure of the butyrivibrio group (Karnati et al., 2009). Protozoal populations probably are inhibited more by combinations of unsaturated fat and increased concentrate than either alone (Firkins, 1996;Firkins et al., 2007). Thus, efforts to explain and predict accumulation of biohydrogenation intermediates must take a broader approach evaluating the interactions between the butyrivibrios and the balanced consortium of fibrolytics, lactate consumers, and protozoa.

Methanogenesis and integration with the consortium

Researchers have been focusing on efforts to reduce methanogenesis because of its potential impact on climate change (Firkins et al., 2008a). Although monensin might decrease methane production, this response might not be accompanied by changes in the archaeal community (Hook et al., 2009) or copies of archaeal rDNA (Weimer et al., 2008). Moreover, if a feed efficiency improvement is a result of slightly decreased dry matter intake (DMI) without affecting milk production (Firkins et al., 2008b), much of the response to monensin for dairy cattle might be independent of a change in VFA pattern (Oelker et al., 2009).

Many researchers have focused on using fat or other additives to inhibit protozoa to reduce the production of H_2. Tea saponins inhibited protozoa but not methane production or copies of archaea 16S rDNA; however, the authors still concluded that the product could be useful to reduce methane production through the archaeal symbionts associated with protozoa (Guo et al., 2008). Although methane production was not affected, the copies of the *mcrA* gene (important in the biochemical pathway for methane production) were reduced dramatically in that study. In another study (Denman et al., 2007), bromochloromethane reduced methanogenesis and the *mcrA* copies proportionately. Decreases in methanogens can be correlated with decreased ruminococci, which produce significant amounts of H_2 (Goel et al., 2009b), so researchers studying partial or complete suppression of protozoa must also consider bacterial populations for their capacities for H_2 production as well as fibrolytic activity. Moreover, a typical approach is to restrict feed intake for all treatments to rule out DMI as a variable (Hegarty et al., 2008), but if fibrolytic activity of protozoa or bacteria is compromised, a decrease in DMI could negate any benefit. Moreover, there could be differences from chemically defaunated animals compared with those born from

defaunated dams (Hegarty et al., 2008). Those using stoichiometric principles to estimate H_2 production need to be aware that H balance can vary tremendously from 100% (Goel et al., 2009b), although this bias might be exacerbated by batch culture compared with a system in which there is a steady state.

Many researchers are using genomic techniques to reduce methanogenesis, but results are not clear cut. Although archaeal sequences clustered predominantly with *Methanobrevibacter* spp. (Wright et al., 2004), supporting objectives to produce a vaccine against *M. ruminantium* to reduce methanogenesis (Williams et al., 2009). However, there was little efficacy and only an increased diversity index. A different inhibitor decreased methanogenesis while also increasing archaeal diversity (Denman et al., 2007). Moreover, these anti-methanogen compounds could increase the partial pressure of H_2 (Kung Jr. et al., 2003). Some predominant archaea that have been selected over thousands of generations to live in the rumen with very low concentrations of H_2 express the *mcrA* gene (with a very low affinity constant) and might not have the gene for the higher affinity isoform (Attwood & McSweeney, 2008). If H_2 accumulates from the inhibitor, populations could shift toward those with a higher Km for H_2. Even methanogens can spill ATP (Russell, 2007) and have growth requirements such as branched chain VFA (Stewart et al., 1997). Thus, methane production rate has previously been shown to be uncoupled from archaeal abundance (Firkins & Yu, 2006). The diversity phylotypes with different methane synthesis pathways, differences of strains within species, and among-animal differences might further complicate this relationship (Zhou et al., 2009). The authors reported that animals with worse feed efficiency had greater abundance of certain archaeal phylotypes, but more work is needed to repeat and extend a mechanism before we can use this information.

Nutrition studies to document reductions in methanogenesis offer an inconsistent benefit. When unsaturated fat (Beauchemin et al., 2009) or coconut oil (Hristov et al., 2009) decreased protozoal numbers without depressing DMI, the decreased methane per unit of DMI could achieve the intended objectives. However, when those fats depressed DMI (Reveneau, 2008;Martin et al., 2008) or decreased digestibility (Goel et al., 2009a), a reduction in the amount of methane produced per unit of DMI might actually be counter-productive because more replacement animals would be needed to meet societal demand for ruminant products (Firkins et al., 2008a). As documented in a recent study evaluating saponins (Holtshausen et al., 2009), decreasing the dosage rate alleviated the negative effects, but it also reduced the efficacy. For industry to adopt these types of feeds and at the proper dosages without unnecessarily high risk for lost profitability, the interactions between all of the microbial populations should be addressed much more holistically in future experiments.

Conclusions

Molecular-based approaches to characterize rumen microbial population structure have undergone a growth period of discovery of untold microbial diversity, but they also have needed continual refinement to reduce biases and increase throughput and objectivity needed for integration with nutrition studies. Samples can be taken, processed, and stored (with proper attention to recovery issues) before deciding which approach best suits the empirical outcomes from the nutrition study. These approaches, with attention to assumptions and limitations described herein, offer considerable potential to help objectively explain the among-experiment variation that limits meta-analyses and computer modeling efforts to improve feed efficiency and reduce environmental impact of ruminant production systems.

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