RUMINANT NUTRITION SYMPOSIUM: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition[1,2]

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ABSTRACT: Metagenomics and high-throughput sequencing have greatly expanded our knowledge of the rumen microbiome. Surveys of all 4 cellular microbial groups (bacteria, archaea, protozoa, and fungi) reveal profound diversity. Even so, evidence exists for core members to perform key degradative or fermentative roles for the host. Some core members are functionally similar yet taxonomically diverse, and noncore members are particularly diverse and probably vary among diets, animals, and over time after feeding. Gains in functional knowledge are being made and offer much potential not only to improve fiber digestibility, decrease methane emissions, and improve efficiency of nitrogen usage but also to help explain the differences in nutrient digestibility or feed efficiency among animals fed the same diet. Integrated research using metagenomics, bioinformatics, traditional ruminant nutrition, and statistical inferences have provided opportunities for ruminant nutritionists and rumen microbiologists to work synergistically to improve nutrient utilization efficiency while minimizing output of wastes and emissions of methane and ammonia. Examples we highlight include residual feed intake, rumen biohydrogenation of unsaturated fatty acids, and dietary inclusion of ionophores. However, there are still some quantitative limitations in approaches being used. This review addresses knowledge gained and current limitations and challenges that remain.

Key words: metagenomics, rumen microbiome, ruminant nutrition

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INTRODUCTION

Krause et al. (2013) described major milestones that improved our understanding of the rumen microbiology-nutrition interface. New technologies have broadened our appreciation of the diversity and complexity of the microbiome. The "core" taxa constitute the primary degraders that have adapted to the rumen and interact with the animal for optimal performance and health (Petri et al., 2013b). A deeper understanding of the entire microbiome, including those taxa that are not core members, is reshaping research questions and hypotheses addressing societal pressures to decrease methane emission or N excretion; improving fiber digestibility, feed intake, and feed efficiency; and decreasing risk for milk fat depression.

Morgavi et al. (2013) discussed genome sequencing projects for ruminal bacteria and archaea, providing useful examples of gains in functional knowledge of the "ruminal superorganism" (i.e., the complex microbiome). They noted coexpression of glycosyl hydrolases with other proteins, such as those used for adherence or for transport of sugars. Although the natural tendency is to add this increasing knowledge of enzymes and fermentation pathways collectively, functional information must be integrated with indices of microbial community

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structure because expression of those genes varies among microbial taxa and feeding conditions. For example, next-generation sequencing (**NGS**) technologies have revealed the importance of poorly characterized ruminal fibrolytic bacteria often exceeding representation from the highly characterized strains of *Fibrobacter* (Jewell et al., 2013) and *Ruminococcus* (Dassa et al., 2014). Thus, much knowledge must be reconciled to continue improving the efficiency of nutrient usage by ruminants. Our aims are to extend recent NGS and metagenomic studies of the rumen microbiome to enrich perspectives, describe challenges, and make recommendations for future research.

DIVERSITY AND FEATURES OF THE RUMEN MICROBIOME

Overview of the Ruminal Microbiome

Ruminal microbes represent a wide taxonomic diversity, including prokaryotes (bacteria and archaea), eukaryotes (anaerobic ciliate protozoa and fungi), and noncellular life (viruses; Hobson and Stewart, 1997). These bacteria, protozoa, and fungi were characterized as different guilds, including cellulolytics, hemicellulolytics, amylolytics, proteolytics, and lipolytics (or, more specifically, the biohydrogenating bacteria), even though many cross over these niches. The large influx of substrates creates selective pressure that subsequently leads to microbial niche differentiation among different dietary conditions and even among different animals. Compared with other microbiomes, the rumen microbiome rather uniquely features low diversity at high taxonomic levels (phylum and class) but high diversity at low taxonomic levels (genus, species, and subspecies). This diversity feature, combined with an opportune supply of substrate and continuous removal of fermentation end products, leads to a high abundance of microbes, which increases the frequency of horizontal (also called lateral) gene transfer and high functional redundancy in which multiple species or subspecies occupy the same niche and perform the same function (Morgavi et al., 2013). Some of the rumen microbes have been categorized to constitute the core microbiome (Lettat and Benchaar, 2013; Petri et al., 2013b; Kim et al., 2014a). Besides this group of common microbes that often are primary degraders (specialists in degradation of complex carbohydrates), other microbes are variable in occurrence and abundance (Wallace, 2008). Intense and intricate interspecies interactions directly influence the performance of ruminant animals, including feed utilization efficiency, output of environmental pollutants (primarily methane and ammonia), and host health (Firkins, 2010).

The abundance of the core and noncore microbes varies because of multiple factors, such as animal species or breed, age and physiological conditions of animals, taxonomic ranks at which microbiomes were compared, feeding regimens, and geographic locations. Individual nutritional studies typically use animals of the same breed on 1 or a few farms to decrease variability and enhance statistical power to detect treatment differences. In that case, the rumen microbiome varies among animals or treatments in relative abundance of its members, rather than complete elimination of existing species or acquisition of new species, because the rumen conditions are more similar compared with conditions among studies (which typically have not been compared). Dietary effects, therefore, influence population dynamics, which need to be reliably and accurately determined by quantitative analysis of populations that can be standardized across studies and allow meta analyses from combined studies. Diversity indices might reflect larger variation among noncore microbes than among the core microbes, so relative abundance of various taxa should be compared. We therefore discuss general foundational knowledge of the cellular microbial groups listed subsequently.

Bacteria

More studies have been devoted to rumen bacteria than to other groups of microbes because bacteria are the major group underpinning feed degradation and fermentation and therefore are the major source of VFA and microbial protein. Cultivation-based studies on rumen bacteria have made important contributions to describing activities of pure or mixed cultures (Dehority, 2003). Although with inherent limitations, cultivation-based studies are needed to definitively determine the metabolism, physiology, and ecology of novel groups characterized on the basis of only rRNA gene sequences (Kim and Yu, 2012; Krause et al., 2013; Creevey et al., 2014). Only 6.5% of the 16S rRNA gene sequences were recovered from cultured rumen bacteria (Kim et al., 2011b). Furthermore, of the 180 bacterial genera identified by 16S rRNA gene sequences, less than 50% (88 genera) of them have a cultured representative. In a recent survey of cultured rumen bacteria from culture collections, scientific literature, and public databases (Creevey et al., 2014), cultured rumen bacteria were noted in 88 existing known genera belonging to 9 phyla, with *Firmicutes* (45 genera), *Proteobacteria* (20 genera), *Actinobacteria* (11 genera), and *Bacteroidetes* (6 genera) representing most of the genera. Only 146 bacterial cultures are archived in 5 major international culture collections. An interesting finding of this survey is that

Bacteroidetes, the second most predominant phylum (in some studies, it was the most predominant phylum), is particularly poorly represented in those culture collections. More effort is indeed needed to isolate members of this numerically and functionally important phylum into pure cultures to enable metabolic, physiological, and genomic characterization.

Methanogenic Archaea

Some strains of archaea, mostly methanogens, were isolated and characterized biochemically and physiologically decades ago. Interest was renewed in the past decade because of the role of enteric methane as a greenhouse gas (Knapp et al., 2014). As with the rumen bacteria, most (>98%) of the archaeal 16S rRNA gene sequences were from uncultured methanogens; however, species richness and diversity of rumen methanogens are much lower than those indices describing bacteria (Kim et al., 2011b). Most of the species clustered in the genera *Methanobrevibacter, Methanosphaera, Methanococcus,* and unclassified *Methanobacteriaceae* (Janssen and Kirs, 2008; Hook et al., 2010). These methanogens generally convert the fermentation products H_2 and CO_2 (or formate) to methane. As illustrated by Knapp et al. (2014), researchers need to be careful when equating H_2 with metabolic hydrogen, which is classically described as **[2H]** because electrons transfer in pairs along with 2 hydrogen atoms to reduce various cofactors such as ferredoxin or NAD. However, when the reduced ferredoxin or NADH + H^+ are reoxidized, intracellular [2H] is converted by membrane-bound hydrogenases to H_2, which diffuses into ruminal fluid. In most cases (except with methanogen inhibitors), little H_2 escapes to the gas phase, and instead, it diffuses back into other cells for hydrogenases to convert H_2 back to [2H]. However, [2H] and H_2 should not be equated because these reactions are among separate microbes and depend on various factors such as thermodynamics of VFA stoichiometry (including various reactions to produce acetate), ruminal pH, and ruminal passage rate (Janssen, 2010). Alternative electron sinks, such as dietary NO_3^- or SO_4^{--}, can be reduced by various bacteria that outcompete methanogens for H_2; thus, feeding these electron sinks need not decrease the acetate:propionate and often increases acetate:propionate (Lin et al., 2013).

Besides demonstrating important interspecies H_2 transfer with *Butyrivibrio proteoclasticus*, Leahy et al. (2010) also noted that *Methanobrevibacter ruminantium* uses acetate as a carbon source for anabolic purposes. Even though there is plenty of acetate in the rumen, most of the methane is produced through the hydrogenotrophic methanogenesis pathway (i.e., using H_2 and CO_2 or formate as substrates, not acetate), with the methylotrophic methanogenesis pathway (i.e., using methanol and methylamines as substrates) contributing to some extent (Carberry et al., 2014). Because of its slow growth rate, the obligatory acetotrophic *Methanosaeta* was rarely detected in the rumen, but the facultative acetotrophic *Methanosarcina*, which can utilize acetate, H_2, and methanol, was detected (Kim et al., 2011b). When grown on acetate as the sole substrate, *Methanosarcina* grows slowly, and increased abundance is noted only in animals with very slow ruminal passage rate (Kittelmann et al., 2013). However, it remains to be determined whether or not ruminal *Methanosarcina* produces methane from acetate through the acetotrophic methanogenesis pathway. Reductive acetogens (or homoacetogens) can use H_2 and CO_2, but they are not as competitive as methanogens under normal ruminal conditions. When methanogens are inhibited during acidosis, however, these acetogens increased their population (Petri et al., 2013b). Moreover, acetogens might be more active in the hindgut of ruminants (Popova et al., 2013).

The ecological and fermentative relationships between methanogens and the other groups (bacteria, protozoa, and fungi) require more research to decrease methane emission sustainably, that is, cost-effectively and without negative consequences on animal productivity (Hristov et al., 2013). Readers are referred to the paper by McAllister et al. (2015) in this issue for details beyond our scope. Methane production was not highly associated with any significant decrease in abundance of methanogens when antimethanogen inhibitors were added in short-term batch cultures of rumen microbes (Patra and Yu, 2014). In a study that did not use antimethanogen inhibitors, no relationship was found between the amount of CH_4 produced and the abundance of methanogens detected in dairy cattle fed 2 diets with different forage-to-concentrate ratios (Danielsson et al., 2012). Methanogen abundance was not associated with CH_4 production in dairy cattle irrespective of supplementation of exogenous fibrolytic enzymes (Zhou et al., 2011). The community composition, rather than abundance of methanogens, was also associated with feed efficiency in beef cattle (Zhou et al., 2010). These studies support our (Firkins and Yu, 2006) earlier synopsis that abundance of methanogens might not be a reliable indicator of methane emission from ruminants.

Multiple factors can affect archaea populations separate from methanogenesis, including the availability of growth factors (Stewart et al., 1997) such as coenzyme M produced by some but not all methanogens (Carberry et al., 2014), shifts between bacterial H_2 producers and nonproducers (Kittelmann et al., 2014), and more

intimate physiological interactions by H_2-producing and -consuming synergistic partners (Morgavi et al., 2013). The inconsistency between methanogenesis and archaea abundance might be related to the within-species expression of genes involved in the methanogenesis pathway (Shi et al., 2014). During methanogenesis, Na^+ is pumped from the methanogen to form a Na^+ motive force such that the resulting Na^+ influx drives both hydrogenases (H_2 converted to [2H]) and ATPase (Buckel and Thauer, 2013). Synthesis of ATP should be driven by a Na^+ gradient but also might be driven by a H^+ gradient when the rumen pH is low (McMillan et al., 2011). Mechanisms for uncoupling methanogenesis with cellular growth are not known but have long been projected (Russell, 2007); one would expect dissipation of Na^+ and H^+ gradients as a way to uncouple ATP synthesis. Also, on the basis of classical Monod kinetics (which are analogous to Michaelis-Menten kinetics for velocity vs. substrate), growth rate likely increases but at a decreasing marginal rate with increasing concentration of aqueous H_2 concentration (Janssen, 2010). Thus, for rapid increases in H_2 production (and aqueous concentration) that occur after feeding (Rooke et al., 2014), methanogenesis per cell might decrease. Clearly, more evaluation is needed to assess archaeal interactions with other members of the microbiome under different ruminal conditions (e.g., varying carbohydrate substrates, time after feeding, and ruminal pH).

Fungi

Anaerobic fungi are present as a minor group in the rumen, but because of their ability to hydrolyze recalcitrant fiber, anaerobic fungi have received much attention from researchers who are interested in harnessing their fibrolytic capabilities (Haitjema et al., 2014). Representatives of 6 genera (i.e., *Anaeromyces*, *Caecomyces*, *Cyllamyces*, *Neocallimastix*, *Orpinomyces*, and *Piromyces*) have been isolated, and some of them have been well characterized with respect to their metabolism and physiology (Gruninger et al., 2014). All of the 6 genera belong to the family *Neocallimstigaceae*, although there is evidence that some isolates or cloned sequences are mischaracterized at low taxonomic ranks (Kittelmann et al., 2012). Phylogenetic analysis of fungal communities in the rumen, based largely on sequence analysis of internal transcribed spacer of the ribosomal gene cluster, revealed the presence of new taxa (Z. Yu, unpublished data). Anaerobic fungi use their hydrogenosomes to anaerobically oxidize pyruvate into acetate plus H_2 and CO_2 or formate (Haitjema et al., 2014). Researchers are evaluating the potential for fungal bioaugmentation to enhance NDF digestibility and alleviate gut fill when animals are fed poor-quality forages. Therefore, increasing fungal activity might increase methanogenesis per animal while decreasing methanogenesis per unit of animal product.

Protozoa

Our foundational understanding of protozoa was accumulated by relatively few researchers who understood the intricacies of protozoal taxonomy, physiology, and dependence on other microbes for growth factors (Williams and Coleman, 1992; Dehority, 2003). Methods to fully or partially defaunate the rumen have been assessed in efforts to improve efficiency of nitrogen usage (Hristov and Jouany, 2005) or decrease methanogenesis (Firkins, 2010), although those authors described caveats to those methods. Analysis of 18S rRNA gene sequences further emphasized the rather unique evolution of rumen protozoa compared with protists in the guts of other herbivores (Moon-van der Staay et al., 2014). Those authors reasoned how ruminal protozoa have benefited from lateral gene transfer to acquire fibrolytic enzymes from bacteria. Ruminal protozoa also have partnered with archaea either as endosymbionts or as exosymbionts (Ushida, 2010) to dispose of the H_2 produced from their hydrogenosomes (Hackstein and Tielens, 2010). An unknown proportion of endosymbionts might be maintained by multiple acquisition rather than dividing synchronously with protozoa (Fenchel and Finlay, 2010).

Diaz et al. (2014) have emphasized that rumen protozoa share many cellular features with other eukaryotic cells, including signal transduction, organelle and vesicle trafficking, and cell cycle control over growth and maintenance; what makes the protozoa in the family *Isotrichidae* (isotrichids) or the order *Entodiniomorphida* (entodiniomorphids, primarily in the family *Ophryoscolecidae*) unique is how they use motility for acquisition of substrate. Those authors derived a model explaining how entodiniomorphid protozoa synchronize their growth rate to keep up with the passage rate of potentially degradable pools of rumen ingesta to which those protozoa are chemotactically attracted. Thus, maintenance in the rumen is not necessarily through strict attachment, although some species (notably *Epidinium caudatum*) momentarily attach to particles to physically shred them (Dehority, 2010) or release enzymes in close proximity (Williams and Coleman, 1992). Nor do entodiniomorphids sediment in the rumen as the isotrichids do (Dehority, 2003). As discussed in Dehority (2003), the isotrichid protozoa clearly establish a migratory ecology toward the dorsal rumen to consume sugars and small starch granules followed by sedimentation to the ventral rumen. They

synchronize these events with feeding; the more feedings per day there are, the more waves of migration by a lower proportion of the isotrichs there are.

Culture-based studies indicated excessive lysis of rumen protozoa. However, those studies did not consider chemorepellence and migration away from lytic conditions (Diaz et al., 2014), which probably are exacerbated under those culture conditions (e.g., feeding once per day and limited stratification of the medium). Those researchers also suggested that protozoal predation of bacteria is likely less in vivo than values measured in batch culture of starved protozoa dosed only with bacteria (i.e., no feed). Protozoal-mediated intraruminal recycling of microbial protein is therefore likely less with high-producing ruminants than expected from in vitro studies (Firkins et al., 2007; Diaz et al., 2014).

Although an argument has been made that methanogens grow slowly and must escape from being washed out of the rumen by endo- or exosymbiosis with protozoa that sequester in the rumen, most archaea in the rumen lack cytochromes and could have division times as low as 1 h (Hook et al., 2010) and have varying strategies for adjusting to ruminal conditions (Attwood et al., 2011). Thus, more strategic consideration of the role of protozoa in the complete rumen microbiome is needed to allow us to partially suppress their biomass to sustainably decrease methane emission per unit of animal production (Firkins, 2010).

From a nutritional standpoint, because biomass, and therefore activity per cell, varies so much among species, including species within the same genus or even strains within species (Whitelaw et al., 1984; Dehority, 2010), we recommend more research to evaluate their biomass, rather than simply relying on cell counts or abundance of 18S rRNA gene copies. Conversion of protozoal 18S rRNA gene copies to biomass requires appropriate attention to collection of a representative protozoal sample that has minimal contamination with bacteria and that is serially spiked into ingesta samples to verify recovery (Sylvester et al., 2005).

CURRENT TECHNOLOGIES FOR ASSESSING THE RUMINAL MICROBIOME

Next-Generation Sequencing

Methods to evaluate distribution or abundance of populations based on DNA have been used in most recent studies (Mackie et al., 2007; McCann et al., 2014a). Initially, 454 pyrosequencing (Roche, Branford, CT) and, now, primarily Illumina sequencing (Illumina, San Diego, CA) have become cost-effective to identify the species present in the rumen microbiome. Many researchers, such as Fouts et al. (2012) and Ross et al. (2012), have attempted to determine both the composition and structure of the rumen microbiome. A group of NGS sequences that share 97% sequence similarity of the 16S rRNA gene is typically clustered into an operational taxonomic unit (**OTU**) because of the difficulty of taxonomically defining unique species of microbes. The relative abundance of an OTU or higher taxon (genus, family, order, class, or phylum) is typically expressed as a percentage of the total number of sequences obtained from a sample, with results from multiple samples being subsequently associated with dietary effects (Callaway et al., 2010; Hristov et al., 2012; Petri et al., 2013a; Ellison et al., 2014).

Although NGS technologies are powerful tools to catalog microbial and gene diversity (including discovery of novel genes), they do not accurately quantify those individual microbial groups or genes (Roh et al., 2010). In nearly all of the studies reported in the literature, NGS data were produced from PCR amplicons. However, PCR amplification is prone to amplification bias resulting from nonspecific annealing or lack of amplification from universal primers (Reysenbach et al., 1992), differential amplification efficiency for different species (Polz and Cavanaugh, 1998), artifact formation (Kurata et al., 2004), or lack of inclusiveness of primers previously thought to be universal (Tymensen and McAllister, 2012). Because the distribution of PCR products can be skewed relative to that of the original template mixture, the prevalence of each OTU (expressed as a percentage of total sequences) might not be indicative of the actual relative abundance of microbes represented by those taxonomically grouped sequences (Brugère et al., 2009). Pooling PCR amplicons from multiple PCR reactions performed at decreased PCR cycles can help reduce PCR bias (Goodrich et al., 2014). Multiplexing of samples using barcodes (Aguirre de Cárcer et al., 2011b) allows a large number of samples (several hundred) to be sequenced simultaneously for subsequent binning with minimal extra cost.

Large numbers of artifactual sequences result from PCR and NGS sequencing, overestimating diversity from about 35% (Gomez-Alvarez et al., 2009; Kunin et al., 2010) to over 600% (Quince et al., 2009). Such problems inevitably complicate analysis from the inherent overestimation but also from the hidden underestimation of the important or true bacterial populations. Although many of the artifactual sequences can be filtered out, it is not possible to filter out all artifactual sequences without losing real sequences. For example, when analyzing 454 pyrosequencing reads, most researchers discard homopolymers longer than 8 nucleotides, assuming them to be artifactual; however, a large number of curated sequences (http://rdp.cme.msu.edu/) produced by the Sanger se-

quencing technology contain homopolymers longer than 8 nucleotides (Z. Yu, unpublished data). In some studies, OTU represented by small numbers of sequences were filtered out on the basis of a probabilistic assumption that these OTU are likely represented by artificial sequences. However, as demonstrated in recent studies that used a mock human gut microbiome of 20 known bacterial species (Kozich et al., 2013; Nelson et al., 2014), no cutoff value (ranging from 0.001% to 0.5% of total sequences) allowed accurate recapturing of the true species composition. Third, sequencing results can be affected by a number of factors, such as DNA template concentration (Kennedy et al., 2014), variation from different sequencing runs and different primers (Kozich et al., 2013; Kim and Yu, 2014), sample storage (Goodrich et al., 2014), and sequence processing and analysis parameters (Z. Yu, unpublished data)**.** Errors relating the quantitative comparison of sequence distributions are likely greater among than within samples (Amend et al., 2010). To our knowledge, no one has used an internal standard to normalize or evaluate sequencing results, although we do note efforts for normalization strategies (Aguirre de Cárcer et al., 2011a). Repeated analyses with multiple clustering approaches were recommended to avoid bias (Goodrich et al., 2014).

Expressing relative abundance of OTU and other taxa as a proportion of their respective totals introduces bias in both directions. A decrease in 1 OTU (or taxon) will increase other OTU (or taxa) and vice versa. Moreover, different numbers of sequence are always obtained from different samples, resulting in different sampling depths and different coverage. Choice of primers (Kim and Yu, 2014), hypervariable region(s) targeted (Engelbrektson et al., 2010; Schloss, 2010), and sequence similarly cutoff values (Kim et al., 2011a) all affect the results of analyses, making comparison of results from different studies difficult.

Quantitative PCR

Sequencing-based methods cannot reliably or accurately quantify a targeted group of rumen microbes based on its DNA signature because of the aforementioned limitations and the lack of a standard or reference that can be used in the analysis. Thus, relative abundance inferred from sequence distributions should be validated by quantitative PCR (**qPCR**). Examples of qPCR application include how dietary intervention affects the relative proportions of select bacterial (Kim and Yu, 2012), archaeal (Zhou et al., 2011), and fungal (Carberry et al., 2012) populations. Also, qPCR has been used to quantify biomass of protozoa (Sylvester et al., 2005) and, subsequently, bacteria and yeast (Castillo-Lopez et al., 2013). Nearly all of the qPCR assays are based on *rrs* (16S or 18S rRNA) genes, except for *mcr*A, which encodes the methyl Co-M reductase that catalyzes the last step of methanogenesis and has been used to quantify methanogens. Because glycoside hydrolases are essential for feed degradation, qPCR analysis of glycoside hydrolase genes can help us understand the kinetics of polysaccharide digestion in the rumen. However, because of the sequence divergence, it is extremely difficult to design glycoside hydrolase family-specific PCR primers. A recent study designed 1 primer set each for glycoside hydrolase families 10 and 11 (both endoxylanases) to examine gene diversity in the goat rumen (Wang et al., 2011). These primers can be used in qPCR analysis of these 2 important glycoside hydrolase families. Future studies may benefit from qPCR analysis of other guilds, such as sulfate-reducing bacteria in cattle fed corn-milling coproducts.

In nearly all of the nutritional or interventional studies, qPCR targeted only a few microbial groups that are perceived to be important on the basis of their status in culture collections. Recently, sequencing-based analysis revealed potentially important unclassified bacteria (Kim and Yu, 2012; McCann et al., 2014b). Future qPCR analysis of these unclassified bacteria in comparative nutritional studies will provide new insight into their role and importance in microbial ecology of the rumen. After noting the high frequency of sequences related to uncharacterized particulate bacteria clustering with *Butyrivibrio fibrisolvens*, Koike et al. (2010) isolated and characterized strains that are likely very important in degradation of hemicellulose on the basis of various enzymatic activities. They subsequently derived primers for qPCR and probes for fluorescent in situ hybridization to characterize their role in the dynamic and spatial colonization of forage. More research like this can help us integrate microbiology and nutrition.

Although qPCR can quantify individual groups of rumen microbes, some of the published primers are not specific and can lead to an overestimate of the abundance. A recent study showed that the primers that have been frequently used in quantifying the genus *Prevotella* (Stevenson and Weimer, 2007) were considered to lack specificity (Bekele et al., 2010). In fact, this primer pair matched nearly 600 non-*Prevotella* sequences from 6 other genera and unclassified bacteria (Kim and Yu, 2012). Improved phylogenetic sampling can invalidate previously validated primers, as was discussed for *F. succinogenes* subgroups (Mosoni et al., 2007). Primers might continue to be challenging to design to be fully inclusive for large and diverse genera yet to be exclusive for all members outside of those genera, or else taxonomic grouping might need refinement (Go-

odrich et al., 2014). We suggest that all researchers periodically reevaluate primers, not against closely related species or genera but systematically against entire databases. Moreover, the specificity of new primers should be experimentally confirmed by analysis of amplicon sequences from actual rumen samples.

Although many researchers normalize 16S rRNA gene copies of target species or groups as a proportion of the total bacterial copies (Stevenson and Weimer, 2007), we offer a few words of caution. For one thing, total bacterial copies can change among treatments (Mosoni et al., 2011), so a change in relative proportion could result from no change in target and just a change in total bacteria. Second, although this approach might normalize for differences in DNA extraction recovery, universal primers can amplify DNA from all taxa of bacteria but can do so with varying amplification efficiencies for different individual species; therefore, a shift in bacterial community structure among samples could bias the relative normalization using universal primers (Smith and Osborn, 2009).

The relative quantification approach of Stevenson and Weimer (2007) accounts for varying DNA extraction efficiencies among samples, and the usage of sample-derived standards for the qPCR assay lessens the opportunity for bias. The formula for relative target copies (percentage of total bacterial copies) uses the efficiency of the PCR reaction and the delta threshold cycle (i.e., the difference of the threshold cycle of control minus the threshold cycle of the sample) for both the target of interest (using taxon-specific primers) and the reference (using universal primers). From the appendix of that report, a simple sensitivity analysis was performed (J. L. Firkins, unpublished data). Each term in the formula was varied by 10% successively to assess the magnitude of change in the formula's solution resulting from each individual 10% deviation; the value of each term was changed to both 90% and 110% of its original value. Varying the efficiency and the delta threshold cycle of the reference had <2% of the potential to change the formula's solution compared with varying the efficiency and delta threshold cycle of the target. Thus, per PCR reaction, nutrition researchers could gain more accuracy (more samples being analyzed) or more precision (more replications of DNA extractions per sample) by using PCR reactions in the plate more strategically for targets. Brankatschk et al. (2012) offered a potentially useful approach to improve qPCR assay logistics. We note the approach of normalizing target 16S rRNA gene copies to the single-copy gene *RNA polymerase* beta subunit (Fernando et al., 2010). One important consideration that needs further validation is whether or not primers targeting the *rpo*B gene are inclusive for all bacteria.

We also performed another simulation in which each value in a random PCR standard curve was changed by a reciprocating 50% on an absolute basis (point 1 × 0.5, point 2 × 1.5, point 3 × 0.5, point 4 × 1.5, etc.) before log normalization (J. L. Firkins, unpublished data). After regressing the modified standard curve, the resulting efficiency changed by only 5%, which is well within the accepted range (Schmittgen and Livak, 2008). The r^2 of the modified standard curve changed by only 1% because large absolute error was diminished by log normalization. Seemingly small deviations in efficiency and r^2 from expectations should be investigated.

Reconsideration of Microarrays

Given some of the quantitative limits of NGS technologies, microarray should be reevaluated. Microarray is a proven powerful technology that enables comprehensive characterization of varying microbiome structures (Paliy et al., 2009) and their metabolic potentials and activities (Wang et al., 2009). Although lacking the depth of NGS platforms, microarrays have several advantages in comparative analysis of complex microbiomes. In addition to microbiome composition, microarray provides uniform, robust, and semiquantitative analysis (linear range up to >5 orders of magnitude) of structure and population dynamics of complex microbiomes (Brodie et al., 2006). Results between replicated analyses can be normalized with internal control or reference standards. A "specialty microarray" can be developed for analysis of microbiomes of interest. Examples of such specialty microarrays include the human intestinal tract chip (HITChip; Claesson et al., 2009; Rajilic-Stojanovic et al., 2009) and the human microbiome chip (HuMiCHipl Tu et al., 2014) for the human intestinal tract microbiome, the pig intestinal tract chip (PITChip) for the gut microbiome of swine (Haenen et al., 2013), and the guild-specific sulfate-reducing prokaryote phylotype chip (SRP-PhyloChip) for sulfate-reducing prokaryotes (Loy et al., 2002). The RumenBactArray enabled semiquantitative comparison of the major bacterial representatives of the rumen microbiome with a linear dynamic range of ≥4 logs (Kim et al., 2014d). Future development of qPCR in array format (e.g., the Custom Microbial DNA qPCR Arrays from Qiagen, Valencia, CA) may enable simultaneous quantification of multiple species or groups of rumen microbes. Because of the conserved nature of *rrs* genes and functional redundancy of the rumen microbiome, *rrs* gene–based microarrays might not fully describe changes in rumen microbial ecology in response to dietary manipulations. The environmental functional gene (micro) array (E-FGA) functional microarray (McGrath et al., 2010) was developed in part as a research aid to control N_2O emissions from soil. As more functional gene sequences of rumen origin are being produced through

NGS (Hess et al., 2011; Wang et al., 2013), a functional microarray might be developed to investigate gene expression of rumen microbiomes in nutritional studies. Moreover, phylochips (e.g., the RumenBactArray) and functional microarrays can be combined so that shifts in microbiome structure and gene expression can be examined simultaneously. Another advantage is that microarray can analyze total RNA, which is more abundant than DNA, eliminating the aforementioned bias associated with PCR or sequencing. Such a benefit would be important in nutritional studies needing accurate quantitative data on the rumen microbiome.

FUNCTIONAL DIVERSITY OF THE MICROBIOME

Surveys of Diversity with Emphasis on Niche and Function

On the basis of a recent examination of the status of phylogenetic diversity census, the rumen was predicted to have, collectively, more than 7,000 species-equivalent OTU of bacteria and nearly 1,500 OTU of archaea (Kim et al., 2011b), which is far more than previously predicted when the number of sequences was much fewer (Edwards et al., 2004). More than 4,000 genus-equivalent OTU were scattered in 19 bacterial phyla, with *Firmicutes*, *Bacteroidetes*, and (to a lesser extent) *Proteobacteria* being the most predominant. In a different survey (Creevey et al., 2014), the number of species-equivalent OTU of rumen bacteria that have been identified worldwide from 16S rRNA gene sequences was lower (2,405 OTU). Differences in analysis methodologies are likely the cause of the above discrepancy. These large numbers of OTU (>2,400) and genera (>190) were reported from an accumulation of individual studies conducted globally that involved a few animals of the same breed per study. Host can have a significant effect on the microbiome structure (Weimer et al., 2010; King et al., 2011; Hernandez-Sanabria et al., 2013; Petri et al., 2013a). Therefore, diet-induced changes (Lettat and Benchaar, 2013), particularly if those changes decrease richness (Mao et al., 2013), could vary as much or more among than within nutrition studies. Standardization of approaches used would increase the utility of comparisons among studies.

The rumen microbiome is skewed not only at the phylum level, with *Firmicutes* and *Bacteroidetes* accounting for more than 90% of the 16S rRNA gene sequences in most of the reported data sets, but also at lower taxonomic levels (Kim et al., 2011b; Creevey et al., 2014). For instance, *Clostridia* and *Bacteroidia* are the most predominant classes, and *Bacteroidales* and *Clostridiales* are the most predominant orders. *Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* are the most predominant families. At the genus level, *Prevotella* is the most predominant in nearly all the rumen microbiomes examined. This might be because of its diverse repertoire of carbohydrate-degrading enzymes (Rosewarne et al., 2014), which supports prior studies documenting the large functional diversity of this genus (Dodd et al., 2009; Bekele et al., 2010; Purushe et al., 2010). Cultured representatives accounted for about one-half (Rosewarne et al., 2014) or even a minority (Kim and Yu, 2012) of the total prevotellas. Therefore, research should prioritize isolation of more species within large and diverse genera, such as *Prevotella*, for functional assessment.

Recent studies also expanded our perspective on the diversity of protozoa (Kittelmann and Janssen, 2011; Ishaq and Wright, 2014), fungi (Kittelmann et al., 2012; Gruninger et al., 2014), and methanogens (Hook et al., 2010; Jeyanathan et al., 2011; Kim et al., 2011b). However, only 1 or sometimes 2 of the 4 groups of rumen microbes were analyzed per study, with bacteria being analyzed most frequently, followed by methanogens. Because of varying technologies used, the interaction among all of these rumen microbial groups is challenging to evaluate across studies. A recent study simultaneously analyzed the 4 groups of rumen microbes by sequencing pooled amplicons prepared separately (Kittelmann et al., 2013). However, the amplicon libraries were not pooled on the basis of the relative abundance of the 4 microbial groups, which prevented quantitative analysis of the actual structure of the rumen microbiome. The contribution and importance of functionally important taxa of the entire microbiome (not just bacteria) need to be assessed more fully to more consistently manipulate digestibility and rumen fermentation profiles toward desired nutritional outcomes.

Advances from Genomic Sequencing Projects and Metagenomics

Knowledge of functional genes or their expression offers the potential to improve fiber digestibility or to explain metabolic variation that occurs with or without major shifts in microbial populations. The genome has been sequenced for several well-characterized rumen microbes, including bacterial strains representing *Butyrivibrio proteoclasticus* (Kelly et al., 2010), *Fibrobacter succinogenes* (Suen et al., 2011a), *Ruminococcus albus* (Suen et al., 2011b), *R. flavefaciens* (Berg Miller et al., 2009), *Oscillibacter ruminantium* (Lee et al., 2012), *Megasphaera elsdenii* (Marx et al., 2011), *Prevotella ruminicola* and *P. bryantii* (Purushe et al., 2010), the methanogen *Methanobrevibacter ruminantium* (Attwood et al., 2011), and a fungal strain of *Orpinomyces* (Youssef et al., 2013). An example of

how genomic information has helped us understand function is the revelation of divergent cellulolytic systems of ruminococci (Dassa et al., 2014).

Metagenomic studies empowered by NGS technologies can be extended to nutritional studies through bioinformatic comparison to annotated genes of cultured and characterized microbes. Either using fosmid or bacterial artificial chromosome clone libraries or using shotgun sequencing, functional gene diversity of the rumen microbiome can be characterized with greater efficiency than sequencing the genome of those individual strains. This area of research was pioneered by Bryan White's group (Brulc et al., 2009), and the promising potential to extensively uncover the functional gene diversity of rumen microbiome is exemplified by the study by Hess et al. (2011). Different categories of genes encoding enzymes involved in feed digestion, fermentation, and microbial protein synthesis (such as carbohydrate metabolism, energy metabolism, amino acid metabolism, and nucleotide metabolism) can be identified by comparison with the Kyoto Encyclopedia of Genes and Genomes (**KEGG**) database (http://www.kegg.jp/), which is a database environment that supports analysis of gene function and metabolic pathways (Kanehisa et al., 2012). New enzymes with novel features have been revealed from rumen metagenomes (Wang et al., 2013; White et al., 2014). Gene clusters organized as polysaccharide utilization loci were first found in *Bacteroides thetaiotaomicron* and were subsequently found in metagenomes from the rumen (Pope et al., 2012; Wang et al., 2013). Gene organizations revealed in a metagenomic study also indicate coordination of feed digestion with other metabolic processes (Wang et al., 2013). Some of the findings through functional metagenomics have greatly expanded our view of the biochemical and metabolic knowledge of the rumen microbiome.

As demonstrated by analyzing the human gut microbiome, if deep sequencing data sets can be produced, forces (either deterministic or stochastic) that shape rumen microbiome structure can be assessed (Jeraldo et al., 2012), and microbiome-level assembly, either co-occurrence or exclusion, can be revealed (Levy and Borenstein, 2013). The metabolic networks of the rumen microbiome, at least those of the predominant members, can also be reconstructed using bioinformatics tools from large metagenomic sequence data sets. Comprehensive databases of metabolic pathways, such as MetaCyc (http://MetaCyc.org; Caspi et al., 2014), KEGG (Kanehisa et al., 2012), and MetaPathways (http://hallam.microbiology.ubc.ca/MetaPathways/) are used to reconstruct metabolic pathways. Although this approach has been demonstrated successfully with a simple microbiome (Mao et al., 2014), it is still challenging to derive meaningful knowledge of metabolism from metabolic sequences of complex and diverse microbiomes, including the rumen microbiome (Caspi et al., 2013; Hanson et al., 2014).

Although metagenomics offers the potential to unearth the metabolic potential of the rumen microbiome, metagenomic data do not provide any information on the gene actually expressed or resultant enzymatic or metabolic activity. For example, the surface area and relative abundance of microbial populations competing for adherence sites probably rate limits fiber degradation, not the abundance of an enzyme per se (Firkins and Yu, 2006; Firkins, 2010), and colonization and enzymatic structure are dynamic (Piao et al., 2014). Metatranscriptomic, metaproteomic, or metabolomic data describing microbial function therefore may be related to the residual composition of feed over time. Quantitative analysis of such data may serve as biomarkers or indicators linking microbial function with nutritional phenotype and animal productivity.

Several technical challenges currently limit the usage of functional metagenomic data in nutritional studies. First, metagenomic sequences produced by the NGS technologies are short reads, ranging from only 200 to 500 bp; sequence assembly to longer gene sequences can produce misassembled genes. This is particularly a problem because of the high diversity at low taxonomic rank at which genes of closely related species or strains share very high sequence similarity. For analysis of phylogenetic diversity, a core set of reference 16S rRNA sequences (DeSantis et al., 2006; Pruesse et al., 2007) can be used to help filter out artificial 16S rRNA gene sequences. To our knowledge, no such core set is available for functional metagenomic data analysis. The metagenomic data sets reported might contain artificial sequences, thus overestimating functional gene diversity. As more microbe genomes are completed, especially the genomes of rumen microbes that are being sequenced through the Hungate 1000 project (http://www.hungate1000.org.nz/), annotation and interpretation of metagenomic data from the rumen microbiome can be greatly facilitated. Because of these issues, microarray is an efficient technology with strong potential to integrate the microbiome's structure and gene expression in ruminant nutritional studies, as demonstrated for the human gut microbiome (Tu et al., 2014).

CONSIDERATIONS FOR ASSESSING THE MICROBIOME IN NUTRITIONAL STUDIES

Persistence of the Rumen Microbiome's Structure among Individual Animals

Differences in rumen microbiome structure among different animals within the same diet group often exceed those observed among different diet groups. Such

interanimal differences are typically explained as a result of different animal genetics and host interactions with the rumen microbiome (Weimer et al., 2010; King et al., 2011; Hernandez-Sanabria et al., 2013). Host effect was demonstrated by gradual return of the rumen microbiome following reciprocal swapping of rumen content of 2 dairy cows (Weimer et al., 2010). That study used automated ribosomal intergenic spacer analysis. The host effect on rumen microbiome now can be better defined by NGS technologies. Unlike the epithelial cells of the gut of animals and humans, the rumen epithelial cells do not excrete mucin or bioactive molecules, such as IgA, antimicrobial peptides, or digestive enzymes. However, the supply of butyrate affects gene expression of the epithelium (Malhi et al., 2013), and the epimural microbes still have important functions for the animal to withstand acidosis (Petri et al., 2013a). Also, the host probably can affect its rumen microbiome in various other ways, such as varying the amount of feed intake, sorting of feed, salivary secretions (affected by forage particle size), and meal-feeding behavior in group housing, all of which can exert physiological effects such as changing frequency of rumination, ruminal volume, stratification of particles, and retention time.

Are the rumen microbiomes, or at least their core members, destined by inoculation at birth? On the basis of terminal restriction fragment length polymorphism analysis, Yáñez-Ruiz et al. (2010) provided evidence that bacterial populations persist (although archaeal populations appeared to be less persistent); consequently, those authors concluded that the adult microbiome potentially could be "programmed" much earlier. Using 454 pyrosequencing of samples from the rumen over time, the microbiome structure shifted with different phases of feeding, but increasing feed intake increased the dominance of sequences that clustered as *Prevotella* species (Rey et al., 2014). In somewhat of a contrast, Li et al. (2012) noted that the rumen microbiome was markedly different with age of calves (coinciding with liquid and solid feeding programs) on the basis of taxonomical characterization, but the microbiome was remarkably similar when assigned on the basis of function. Use of NGS begs the question regarding the initiation of the core microbiome and its persistence after dietary shifts (Petri et al., 2013b). Although the microbiome at initial colonization or during key phases may differ with later stages, the prior microbiome structure still might influence the structure of the subsequent (and different) microbiome (Jami et al., 2013).

The Microbiome and Residual Feed Intake

In several recent studies, associations were detected between a few microbial groups and animal production and feed efficiency, with some potential associations drawn to ruminal fermentation. Residual feed intake (**RFI**; decreasing RFI means the animal is increasingly more efficient than predicted) was associated with microbial groups on the basis of denaturing gradient gel electrophoresis (Carberry et al., 2012; Hernandez-Sanabria et al., 2012). Concentrations of VFA were associated with the abundance of the lactilytic *Selenomonas ruminantium* and *Megasphaera elsdenii* in the rumen of dairy cows during the transition period (Wang et al., 2012); those bacteria help to prevent accumulation of lactate even in subacute rumen acidosis (**SARA**) challenge models (Mao et al., 2013). The relative proportion of sequences that were assigned to *Prevotella* appeared to be positively associated with high RFI in bulls, whereas a group that was unidentified but within the order *Bacteroidales* was positively associated with bulls that had low RFI (McCann et al., 2014b). *Prevotella* was negatively associated with RFI and milk fat production in dairy cattle (Jami et al., 2014). The proportion of sequences assigned to *Prevotella* has been associated variably with rumen acidosis (Golder et al., 2014b). As described previously, variable responses are likely a result of the large diversity within the *Prevotella* genus (Jami and Mizrahi, 2012) and probably await more stringent validation, perhaps with more robust qPCR or microarray approaches. However, we note that readers need to be careful of simple correlation analyses for inferences drawn unless the data are dispersed over the entire range; 1 high correlation ($r = 0.88$) between the proportion of *Fibrobacter* sequences in the total bacterial sequences recovered from NGS vs. relative qPCR among different animals was driven primarily by 2 of the 21 points; if those 2 greatest abundances were removed, the correlation decreased to $r = 0.05$ (J. L. Firkins, unpublished data). This analysis begs the question why would there be such a low abundance of the prodigious fibrolytic bacterium, *F. succinogenes* (Ransom-Jones et al., 2012; Jewell et al., 2013)? On the basis of assumptions for >10% abundance of primary cellulolytics (Russell et al., 2009), are low recoveries of sequences representing known cellulolytics an artifact of sampling (e.g., underrepresenting the fibrous rumen mat) and analysis procedures (discussed subsequently), or are they a real phenomenon associated with feeding mixed forage-concentrate diets to dairy cattle at high feed intakes and with postprandial fluctuation in ruminal pH?

Spearman rank correlation tests the association between abundance (or relative abundance) of individual microbial populations that correlate with variables of rumen fermentation and animal performance, whereas canonical correspondence analysis can be used to analyze associations between microbiome structure and individual variables of animals (Carberry et al., 2012). In doing so, one can make inferences for the groups

of microbes that associate with a given measurement with certain caveats. First, ruminal microbiome data have been infrequently associated with data on ruminal fermentation or animal performance. Second, the *Firmicutes* to *Bacteroidetes* ratio has been used to examine microbiome structure (Pinloche et al., 2013), and in another study, this ratio was positively correlated with milk fat yield (Jami et al., 2014). Readers should be aware of statistical problems associated with ratios derived from correlated variables (Kim, 1999). That author recommends fuller models using multiple regression rather than a condensed model based on a ratio. Quantitative information might be more robust if it is based on a set of "key" or "indicator" microbes or even based on aggregated groups of microbes (perhaps grouped using principal component analysis). Goodrich et al. (2014) noted many opportunities to account for variation in NGS methods, including various statistical approaches. Those authors also noted that data do not always follow a normal distribution, requiring transformation or use of nonparametric statistics. Some multivariate approaches force linear associations to some relationships that are inherently nonlinear (Firkins, 2010). Thus, approaches to improve the rigor of NGS data analysis to assess the microbiome structure within a sample should have the same rigor extended for statistical integration of those results across multiple samples and with other measurements from nutritional studies. No single statistical approach fits all conditions, and readers are encouraged to seek both bioinformatics and statistical support before initiating these complex studies.

Metabolomics approaches are now being used in nutritional studies (Saleem et al., 2012; Zhao et al., 2014). Kingston-Smith et al. (2013) revealed that forages of different genotypes that are apparently similar by traditional nutritional analysis produced different patterns of metabolite flux. As metabolomic technologies continue to improve in separation and identification and become more cost-effective, there is potential to discover metabolic biomarkers and thereby derive more powerful associations between varying animal responses to dietary variables and quantitative measures describing the structure and function of the rumen microbiome. Future analyses need to include more measurements besides VFA profiles. The VFA are net outcomes from potentially varying rates of production, interconversion, and absorption. Other measures might include nitrogenous fractions, bacteriocins, or enterotoxins. Enzymatic activities would be complemented with approaches to estimate feed digestibility.

Finally, we need to be aware that not all variations will be explained deterministically. Our preliminary data (P. Kongmun, M. Wanapat, and Z. Yu, unpublished data) demonstrated that the structure of the rumen microbiome also undergoes stochastic drift, which might be a result of random shifts of core members of the microbiome that fill a similar function but are distantly related taxonomically or else may result from oscillation among the noncore microbes (see previous discussion). Therefore, adequate observations over time, among animals, and perhaps even at different locations within the rumen are needed to account for random fluctuations.

How, Where, and When to Sample?

Most of the early studies using molecular approaches to study rumen bacterial populations collected fluid and ignored the more important particulate fraction (Firkins and Yu, 2006). As much as 92% of the total bacterial 16S rRNA gene copies was estimated to be associated with the particulates (Mullins et al., 2013), although such a characterization depends on whether or not the loosely adherent bacteria are excluded (Larue et al., 2005). Mullins et al. (2013) emphasized the importance of harvesting particulate bacteria for estimating outflow of bacterial biomass. Because of the difficulty in dislodging the tightly adherent bacteria (Whitehouse et al., 1994), we agree with Mullins et al. (2013) that particulate bacteria should be extracted for collection of bacterial standards for flow measurements but not for analysis of the rumen microbiome, for which DNA should be extracted from the entire sample rather than from previously harvested microbial cells.

Sampling of rumen fluid through stomach tubing is a practical means to achieve samples from the large number of animals needed to assess the microbiome in a practical setting. Evaluation of the liquid phase probably offers considerable potential to improve our understanding of events leading to ruminal acidosis, although inferences related to the particulate bacteria must be evaluated with caution (Golder et al., 2014a). Using only *Streptococcus bovis* and *P. ruminicola* as indicators, the use of a stomach tube provided equivalent responses compared with samples collected through a rumen cannula for calves (Terré et al., 2013). Those authors noted a greater concentration of VFA in samples from the rumen cannula, which should be expected on the basis of the greater production rate of VFA in the mat compared with the slower distribution of those VFA to the surrounding layers for absorption (Storm et al., 2012). Many of the amylolytic bacteria probably also adhere to grain particles, and sequencing approaches have documented a greater importance for amylolytic ruminococci that adhere to feed particles compared with prior expectations (Larue et al., 2005; Klieve et al., 2007). Thus, sampling from the fluid phase vs. particulate phase in the rumen mat depends on nutritional objectives. In 1 study, 24 rumen-cannulated cows

were used (Golder et al., 2014b); if that many cannulated animals cannot be used in a single study, researchers should consider compiling samples across different studies using standardized methods (discussed previously) to achieve the divergence and statistical power needed for more appropriate characterization of dietary interactions with the rumen microbiome. As often assessed through a rumen cannula, the potential role of the epimural bacteria (Chen et al., 2011) needs further attention, particularly if these microbes are influencing gene expression of rumen epithelial cells.

Some researchers such as Li et al. (2009) and Golder et al. (2014a) have minimized the importance of postprandial sampling time. However, conclusions derived from such studies must be considered within the context that 1) rumen samples contain both newly ingested and previously ingested feed and 2) subsequent progression of a developing consortium of microbes degrading newly ingested particles is dynamic; the ultimate microbial structure might be dictated by early colonizers that subsequently become diluted by that developing consortium (Shinkai et al., 2010). Firkins (2010) already extensively discussed the importance of a balanced consortium of colonizing bacteria to maximize fiber digestibility in the rumen, and metagenomic approaches have confirmed the sequential degradation of forage fiber (Brulc et al., 2009; Li et al., 2013; Piao et al., 2014). These responses are often explained or confirmed on the basis of sequential changes in bacterial 16S rRNA gene copies (Mullins et al., 2013). In contrast, researchers also should consider potential roles for microbes among different locations of the particulate fraction (Shinkai and Kobayashi, 2007) or even differential gene expression over time without necessarily changing the bacterial composition, as shown for *F. succinogenes* (Béra-Maillet et al., 2009). Studies assessing the abundance of *F. succinogenes* might not cover the full diversity of this species but still reflect relative effects of concentrate feeding (Mosoni et al., 2007). For these reasons, more attention to the dynamic response of microbial colonization and fiber degradation may help explain more differences in ruminal digestibility than evaluating the microbiome on the basis of pooled samples. This contention is further supported by the addition of yeast (which does not degrade fiber) to influence the ruminal microbiome structure and decrease susceptibility to negative associative effects associated with an unbalanced consortium (Pinloche et al., 2013).

Microbial Effects on Biohydrogenation and Milk Composition

Considerable progress has been made in our understanding of biohydrogenation (**BH**) and groups of bacteria responsible for BH (Jenkins et al., 2008). Pathways of BH are much more complex than previously thought (McKain et al., 2010; Lee and Jenkins, 2011). Some of the divergence among pathways might involve plant factors influencing lipolysis and BH (Kim et al., 2009) or processing factors, especially heating (Kaleem et al., 2013). Some shifts in BH pathways have been associated with changes in population abundance of key bacteria, such as *Clostridium proteoclasticum*, which represents the minority of bacteria that fully hydrogenate 18:1-*trans* isomers to 18:0 (Kim et al., 2008). In contrast, differences in BH indices could not be associated with population changes of various bacterial targets (Halmemies-Beauchet-Filleau et al., 2013). A recent study (Petri et al., 2014) illustrates the strengths and weaknesses of NGS for evaluating ruminal BH. Clear shifts in key biohydrogenating bacteria were associated with the fatty acid profile of adipose tissue in steers; however, further inferences were limited by the methods to differentiate among those closely related biohydrogenating bacteria.

The ratio of forage to concentrate could influence the relative abundances of the biohydrogenating bacteria compared with those that do not biohydrogenate but compete with biohydrogenating bacteria for the same carbohydrate substrate (Zened et al., 2013a,b). Various dietary regimens influencing sequence distributions within and among various taxa were discussed, including potential differences among studies resulting from sampling location (fluid vs. particles) and PCR bias (Zened et al., 2013a). The abundance of *Megasphaera elsdenii* is associated with the formation of BH intermediates that promote milk fat depression (Palmonari et al., 2010), whereas its role in producing 18:1 *trans*-10 was challenged (Maia et al., 2007). In the latter study, increasing lactate concentration increased the sensitivity to linoleic acid by several biohydrogenating bacteria. Post hoc associations are not cause and effect. An alternative research question to whether *M. elsdenii* promotes milk fat depression as assessed by an inverse correlation with milk fat is, in contrast, whether or not *M. elsdenii* may not have increased enough to prevent milk fat depression. This bacterium is well known for its role in lactate uptake and metabolism (Nagaraja and Titgemeyer, 2007). When ruminal fluid from cows that were not milk fat depressed was transferred into those that had been induced into milk fat depression, the time needed for recovery was decreased (Rico et al., 2014). This study emphasizes the need to understand further why some cows are resilient against milk fat depression and to understand how to hasten recovery from disbalanced population structures and the interacting dietary variables that shift BH patterns from those cows that have bouts of milk fat depression.

Biohydrogenation of PUFA is not likely a major sink for reducing equivalents produced during fermentation, so BH is primarily a defense against PUFA toxicity (Jenkins et al., 2008). This toxicity is generally considered more severe with increasing intake of starch but not sugar under moderate inclusion rates (Martel et al., 2011). Toxicity of PUFA is more likely a result of metabolic interruption (depressed intracellular ATP and acyl CoA pools) rather than membrane toxicity (Maia et al., 2010).

Protozoal predation may also influence populations of the butyrivibrios and related groups involved in BH (Karnati et al., 2009). Protozoal membranes are a depot for PUFA and partially hydrogenated FA that keep those FA out of the BH pool (Firkins et al., 2008), so using dietary fat to inhibit protozoa (to decrease methane emission or urinary N excretion) could increase risk for milk fat depression. Protozoa can be inhibited by medium-chain fatty acids differentially (Reveneau et al., 2012a), and numerous other microbial populations can be affected (Hristov et al., 2012). Thus, future researchers need to consider the entire microbiome when assessing ruminal PUFA metabolism.

Although PUFA may exert bacteriostatic effects by disrupted metabolism (Maia et al., 2010), medium-chain fatty acids have been shown to disrupt membrane function and K^+ gradients in archaea, which have cell membrane and wall structures dissimilar from those of bacteria (Zhou et al., 2013). Although offering promise to inhibit protozoa and archaea, lauric acid can depress DMI and milk fat production (Hristov et al., 2011; Reveneau et al., 2012b). Lauric acid increased *Methanosphaera elsdenii* at the expense of *Methanobrevibacter* species of archaea while it concomitantly increased certain Gram-positive bacterial genera at the expense of Gram-negative bacteria (Hristov et al., 2012). As shown for lauric acid, more mechanistic studies assessing the entire microbiome are needed to fully understand the mechanisms of differential susceptibilities of other bioactive feed ingredients being developed.

Microbial Adaptation from Acidosis or Monensin

Nagaraja and Titgemeyer (2007) eloquently described foundational principles related to SARA in beef cattle. Some dairy cows are more prone to SARA than other dairy cows (Mohammed et al., 2012), presumably at least in part because of varying microbial structure. As discussed previously, an acidosis challenge shifted the structure of the rumen microbiome (Golder et al., 2014a). Various microbial populations can adapt to tannins (Smith et al., 2005), essential oils (Patra and Saxena, 2009), and monensin (Lima et al., 2009) by changing their membrane or cell envelope. Thus, adaptation to SARA may involve changing the cell membrane's architecture of some taxa to counter the lower pH. More information is needed to explain such complex shifts (not just of a few members such as *Streptococcus bovis* and *Megasphaera elsdenii*) associated with different animals and dietary variables that promote or resist the succession of microbial populations before those challenges (Petri et al., 2013a).

Monensin and other ionophores have several benefits, including promoting growth, improving feed efficiency, improving the efficiency of nitrogen usage, decreasing the risk of rumen acidosis of beef cattle fed high-grain diets, and decreasing methane emission (McGuffey et al., 2001; Ipharraguerre and Clark, 2003; Beauchemin et al., 2008). Dietary interactions with monensin deserve further testing for beef cattle (DiLorenzo and Galyean, 2010) as do the other ionophores approved for use in beef cattle. The mode of action of ionophores and bacterial adaptations to them have been described (Morehead and Dawson, 1992; Callaway et al., 2003). Gram-positive bacteria, which lack a protective outer cell membrane, are generally more susceptible to monensin and similar ionophores than Gram-negative bacteria are. Comprehensive studies on the effect of monensin on rumen microbiome using NGS also showed that monensin can decrease the relative abundance of some Gram-positive bacteria while decreasing the relative abundance of some Gram-negative bacteria, but the major change has been within those groups (Kim et al., 2014b,c). Protozoa also adjust to monensin, although the adaptation appears to be mediated by changes in intracellular membrane composition (Sylvester et al., 2009). Only monensin is approved for beef cattle and lactating dairy cattle in the United States. Although ruminal acetate:propionate generally declines with feedlot beef cattle fed monensin (Ellis et al., 2012), results are more variable with lactating dairy cattle (Appuhamy et al., 2013). Reasons were attributed to greater DMI, increased forage for dairy cattle compared with feedlot cattle, and perhaps increasing persistency of longer-term usage for dairy. Researchers should also consider the potential role for monensin to change feeding behavior (González et al., 2012), which could indirectly influence ruminal fermentation. Although monensin has varying effects on acetate:propionate, it still maintains persistent improvements in feed efficiency for both beef and lactating dairy cows. Thus, one of the most historically important nutritional interventions on the microbiome still deserves further research attention.

SUMMARY AND CONCLUSIONS

The current understanding of the diversity and complexity of the rumen microbiome greatly exceeds our understanding before the widespread usage of metagenomics and NGS approaches. With this technological clout comes a renewed obligation to partner with researchers who can derive mechanistic results from functional genomics, transcriptomics, physiology, and the ecology of uncharacterized microbes along with improved bioinformatics and statistical association of microbiomes among different animals with different feeding regimens. Considerable opportunities abound to continue expanding our understanding of the rumen microbiome and its role in ruminant nutrition.

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