A Microbiologist's View on Improving Nutrient Utilization in Ruminants

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Introduction

Ruminants, particularly cattle, sheep, and goats, are important production animals for food to humans worldwide. Their importance comes from their unique ability to convert, because of foregut microbial fermentation, fiber-based feeds with or without grains, into high quality, protein-rich products like milk and meat. The rumen, the first compartment of the complex stomach, is inhabited by a multitude of microbes that work in concert to breakdown feeds to produce energy (volatile fatty acids; **VFA**), protein (microbial cells) and other nutrients like vitamins (microbial cells) to the host. The production of VFA, mainly from carbohydrates. is central to the ruminal fermentation because the process provides energy (**ATP**) for microbial growth, which serves as the major source of protein to the host, but also provides the animal with the precursors necessary to generate energy (mainly acetate), glucose (mainly propionate), and lipid (mainly acetate and butyrate). The fermentation of nitrogenous compounds is also an integral process because it provides the molecules necessary to build microbial cell protein.

In addition to the importance of the rumen microbial function to the host nutrition and food production, rumen microbes and their enzymes are also of considerable interest to the biofuels and biotechnology industries (Hess et al., 2011). Despite the tremendous importance, rumen remains an under investigated, hence, under-characterized, microbial ecosystem. At one time, rumen was the most extensively investigated anaerobic ecosystem. In the past 10 to 12 years, human gut microbial studies have far outpaced rumen microbiology studies. The human gut microbiome studies were part of the National Institute of Health-funded Human Microbiome Project, a logical extension of the Human Genome Project, to study the distribution and evolution of the constituent microorganisms in the human body (Turnbaugh et al. 2007; Llyod-Price et al., 2016). The impetus for the gut microbiome studies is largely because of the profound impact that gut microbes have been shown to have in human health and diseases (Cani et al., 2018). The explosive growth in the study of gut microbes is because of the development of high-throughput and high-resolution molecular methods to unravel the community composition and functional role in the ecosystem.

Molecular Methods ("Omics" Approach) to Delineate Ruminal Ecology and Function

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Initial molecular techniques were based on amplification of nucleic acids by PCR, both conventional and real-time, and restriction fragment length polymorphic analyses, such as ribotyping, pulsed-field gel electrophoresis, denatured gradient gel electrophoresis for identification and genetic typing. In recent years, research on rumen microbial ecology has expanded and exploded because of high-throughput and high-resolution nucleic acid sequence and chemical separation and identification methods for protein and metabolites analyses. The advances in nucleic acid sequencing and bioinformatics analyses (Amplicon sequencing and Metagenomics) have enabled researchers to analyze community composition and function by culture independent methods. DNA sequence information provides insight into microbial community composition ('who are there'), but does not provide a direct measure of the function ('what are they doing?'), although potential function can be deduced from the genes identified. Therefore, analyses that measure gene expressions or transcription of DNA to messenger RNA (**mRNA**), called (meta)transcriptomics, translation of mRNA into protein, called (meta)proteomics, or ultimately production of products or metabolites, called metabolomics, are necessary to delineate functional profiling of the microbial community in the rumen.

Amplicon Sequencing and Metagenomics. Sequence-based taxonomic profiling of a microbiome are carried out by amplifying 16S rRNA genes or by whole-metagenome shotgun sequencing. Amplicon sequences of 16S rRNA (reads) are commonly grouped into clusters, called as 'operational taxonomic units (**OTUs**)', which are then assigned to specific taxa based on sequence homology to a reference genomic sequence. In shotgun metagenomics, sequencing methods are applied to millions of random genomic fragments of DNA extracted from ruminal contents. The shotgun sequence reads are used to determine community composition, either by considering the reads individually or by first assembling them into contigs, which are then compared to a reference catalog of microbial genes or genomes. Such community analyses allow researchers to carry out taxonomic profiling of the microbial community to answer the question, 'who are present?' in the rumen. Taxonomic profiling of microbial species in the rumen have been performed on the different ruminant species (cattle, sheep, goats, and buffaloes) in relation to animal to animal variation, diet changes, ruminal disorders (acidosis, bloat, milk fat depression), feed efficiency, milk production, methane production, maternal influence, feed additives, and seasonal changes, etc. (Denman et al., 2018; McCann et al., 2014). The utility and applicability of the rumen microbial profiling by molecular techniques are best evidenced by a study published by Henderson et al. (2015). The study to assess the effects of diet, animal species and geographical location on ruminal microbial population involved 742 ruminal content samples from 32 animal species located in 35 countries. The differences in microbial communities were predominantly attributable to diet, and host factors were less influential. The protozoal communities were variable, but dominant bacteria and archaea were similar among all samples, and across animal species, diet, and geographical region a core microbiome was present (Henderson et al., 2015).

Metatranscriptomics. The metatranscriptomics, also called RNA-seq, involves sequencing all of the RNA produced by a microbial community, except ribosomal RNA,

which is first depleted before sequencing. The RNA preparation is essentially mRNA, which is converted to DNA, called complementary DNA (**cDNA**), for sequencing. A few of the studies on metatranscriptomics have focused on carbohydrate-degrading enzymes associated with microbes adherent to the fiber (Dai et al., 2015; Comtet-Marre et al., 2017). These studies have confirmed culture-base studies that major bacterial activities of fiber degradation were associated with species of the genera *Fibrobacter, Prevotella* and *Ruminococcus*, but also indicated large contribution of fungal and protozoal species.

Metaproteomics. Measuring protein abundance provides a more direct indicator of the functional activity of the microbes. The high-throughput method of measuring proteins and their abundance, called metaproteomics, involves mass-spectrometry-based shotgun quantification of peptide mass and abundance. The peptides are then associated with full-length proteins by sequence homology-based searches against reference databases, similar to data bases available for DNA and RNA sequences. Studies on metaproteomics of ruminal fluid are limited (Snelling and Wallace, 2017; Deusch and Seifert, 2015). The study by Deusch and Seifert (2015) identified in excess of 2,000 bacterial, 150 archaeal, and 800 fungal and protozoal proteins in the fiber adherent fraction of the ruminal digesta.

Metabolomics. The metabolomics refers to the detection, identification, and often quantification of metabolites and other small molecules in microbial communities. It is not done by predictions based on genomic information, instead, the analysis relies on techniques, such as high performance liquid chromatography, to separate chemicals, which are then identified and quantified by mass spectroscopy. Ruminal VFA analysis, a widely used technique in ruminal fermentation studies, is an example of a metabolomics. However, metabolomics is a more comprehensive chemical analysis that detects and quantifies all possible chemicals present in a sample. The first study on metabolomics of ruminal fluid was published by Ametaj et al (2010). The study measured ruminal metabolites of dairy cows fed diets with increasing proportions of grain. The results showed unhealthy alterations in the metabolites (increased methylamine, dimethylamine, N-nitrosodimethylamine, endotoxin, ethanol, phenylacetylglycine, etc.) in ruminal fluid of cows fed higher amounts of grains. What is not known how these alterations are linked to ruminal dysfunction.

Genomics of Ruminal Microbes

Genomics is the science of sequencing, mapping, and analyzing the entire complement of genetic information of an organism. Essentially, it is a genetic blueprint that provides complete information on the evolution and physiology of the organism. The process provides raw sequences that need to be assembled and annotated (read) to provide biological meaning. The process has become so inexpensive and common, the technique has become routine and often a starting point for characterizing and analyzing the metabolic potential of an organism. The first rumen bacterial species that was genome sequenced was *Fibrobacter succinogenes,* a dominant fibrolytic bacterium (Jun et al., 2007). A global project on a comprehensive genomic analysis of ruminal microbes has been initiated, somewhat similar human gut microbiome project. The Hungate 1000

project (www.Hunagte1000.org.nz or http://www.rmgnetwork.org/hungate1000.html), a global initiative launched in 2012, was designed to provide a reference set of rumen microbial genome sequences from cultivated ruminal bacteria, archaea, fungi and ciliated protozoa. The database, which are publicly available, will enable researchers to analyze the physiology and metabolic potential of the organism with regard to ruminal function. At the beginning, genome sequences were available for 14 bacterial species (belonging to 11 of 88 known genera in the rumen) and one methanogen. Currently, 501 organisms (belonging to 73 of 88 genera) have been sequenced, referred to as Hungate genome catalog (Seshadri et al., 2018). Anaerobic fungal genomes have been difficult to sequence because of their high adenine and thymine content, repeat-sequences, complex physiology and unknown ploidy (Edwards et al., 2017). So far, whole genomes of five fungal species have been sequenced and are publicly available; however, there are no genomic sequence data on ciliated protozoa of the rumen.

Ruminal Microbes and Nutrient Utilization

A simple microscopic examination of ruminal fluid reveals a complex and diverse microbial population. The population includes members of all three domains of life: Bacteria, Archaea (methanogens) and Eukarya (fungi and protozoa). The fermentative activities of these microbes convert complex organic feedstuffs into mainly volatile fatty acids and microbial protein, which are then used by the host for growth and production. Of the three domains, bacteria are the dominant population and most extensively investigated. Additionally, as in most microbial ecosystems, rumen also possesses subcellular organisms called bacterial viruses or bacteriophages. The structure and contribution of the viral community is the least investigated and hence not much is known about their role.

Ruminal Bacteria. Rumen is inhabited by a dense population of bacteria (up to 100 billion per g of contents). They are broadly categorized into fluid-associated, solids-associated, and eukaryotic cell-associated, with the majority of the bacteria associated with the solids (up to 80% of the total). The eukaryotic cell-associated bacteria include those adherent to ruminal epithelial cells, protozoa, and fungi. The bacteria attached to epithelial cells, called epimural bacteria, do not contribute to digestion of feeds. Before the advent of molecular techniques, the understanding of the ruminal microbial ecology and its contribution to the host nutrition was based on classical culture methods (fancily called culturomics!), pioneered by Robert Hungate (the father of rumen microbiology) and his student, Marvin Bryant. Many of the extensively studied bacterial species in the past 60 years (species of bacteria belonging to the genera *Butyrivibrio, Fibrobacter, Lachnospira, Megasphaera, Prevotella, Ruminococcus, Selenomonas, Streptococcus* etc., just to name a few) are likely in high abundance, hence easily isolated and characterized. They possess a multitude of enzymes (amylases, cellulases, hemicellulases, lipases, proteases, etc.) that contribute to digestion of starch, fiber, lipids and proteins in the rumen. The major products produced by bacterial fermentation include acetate, propionate, butyrate, lactate, H_2 and CO_2. In subsequent years, the culture methods have evolved to isolate and quantify a number of bacterial species by utilizing general purpose and selective culture media and characterize the isolates with regard to

their fermentative activities and production of end products. The culture methods have identified several genera and species, categorized broadly as ‘generalists’ and ‘specialists’. In recent years, there is more emphasis on the culture-independent methods, which have provided identity and quantity of microbes and have vastly expanded our understanding of the community composition. These studies have identified novel genera and a number of them have not been cultured (*Acetivibrio, Allobaculum, Anaerobaculum, Anaerophaga, Blautia, Eggerthella, Howardella, Mogibacterium, Moryella, Peptinophilus, Proteocatella, Robinsonella, Tissierella, Victivalis*, etc., just to name a few). In a recent study to compare culture methods and culture-independent methods, only 23% of the bacterial types identified by molecular methods were captured by cultured methods. The use of multiple media increased the number of cultured bacteria to 40% (Zehavi et al., 2018). Regardless, molecular methods to detect bacterial community composition have indicated that a majority of ruminal bacteria have not been cultured, therefore, nothing is known about their role in ruminal fermentation.

Ruminal Archaea. The abundance of archaea in the rumen is estimated to be about 5% or less of the total microbial mass. The archaeal domain in the rumen is the methanogens that produce methane, which is eructated and released into the environment. Methanogens exist in all anaerobic ecosystems and as many as 113 species in 28 genera have been described, however only a few have been cultured from the rumen (McAllister et al., 2015). Similar to the rumen bacterial population, a core community of methanogens exist in the rumen (Henderson et al. 2015). The primary methanogens in the rumen are hydrogenotrophs, which produce methane by reducing CO_2 (hydrogenotrophic pathway). Hydrogenotrophic methanogens include the genus *Methanobrevibacter,* which is subdivided into the SMT clade (*M. smithii, M. millerae, M. gottschalki, M. thaurei*) and RO clade (*M. ruminantium, M. olleyae*). *Methanobacter ruminantium* is the dominant species in the rumen. The less abundant methanogens, called methylotrophic methanogens, reduce from methyl group of substrates like methanol and methylamines (methylotrophic pathway). The acetoclastic methanogens that produce methane from acetate (acetoclastic pathway) are in low numbers in the rumen, but are abundant in all anaerobic ecosystems other than the gastrointestinal tract (Morgavi et al. 2010). In addition to free-floating methanogens in ruminal contents, there are additional niches in the rumen, which include association with the ruminal epithelial cells and in symbiotic associations with H_2 -producing protozoa and fungi. In ciliated protozoa, methanogens exist inside the protozoan cell as endosymbionts and on the surface as ectosymbionts (McAllister et al., 2015).

Ruminal Ciliated Protozoa. Protozoa represent up to 50% of the microbial mass in the rumen. The dominant protozoa in the rumen are ciliated or flagellated, and the flagellates are in low numbers, hence, functionally not significant. Ciliated protozoa are the most readily visualized microbe microscopically in the rumen because of their size and distinct morphological characteristics. A negative aspect that distinguishes ciliated protozoa from ruminal bacteria and fungi is that it is almost impossible to grow them outside the rumen (*in vitro*) and maintain them in pure culture, which has limited our knowledge on their physiological characteristics and contributions to ruminal

fermentation. Therefore, a large number of studies on protozoa are based on microscopic identification and enumeration, at genus and or species level, and by elimination from the rumen, a process called defaunation, using a variety of chemical and physical methods. However, defaunation is not easy to accomplish and maintain. Molecular techniques that have allowed cloning and expression of protozoal genes (for example, genes that code for fibrolytic enzymes) have allowed identification and characterization of a few enzymes (Newbold et. al., 2005). Such observations have been confirmed by metagenomic analysis of genes that code for enzymes involved in carbohydrate fermentation (for example, glycoside hydrolases) (Findley et al., 2011).

Although ciliated protozoa contribute to digestibility of feeds and VFA production, their overall role in ruminal fermentation and contribution to the host nutrition is still an area of considerable debate and controversy (Viera, 1986; Newbold et al., 2015). Much of the debate on the role of ciliated protozoa is on the amount of protozoal flow to the lower gut and their contribution to the protein supply to the host. Based on microscopic counts of protozoa in postruminal contents, counts account anywhere from 6 to 64% of ruminal fluid counts (Viera, 1986; Puniya et al. 1992). However, Sylvester et al (2006) have reported, based on real time PCR assay, that post-ruminal flow of protozoa is proportional to the ruminal protozoal mass. It is well known that ciliated protozoa are not essential to the ruminal fermentation and host nutrition based on defaunation studies. The effects of defaunation include changes in physical and chemical characteristics of the ruminal environment. A meta-analysis on the main effects of defauantion based on 23 in vivo studies comprising 48 comparisons (Newbold et al., 2015). A majority of the studies were done in sheep (87%). Defaunation increases microbial protein supply (up to 30%) and decreases methane production (up to 11%). Because protozoa predate on bacteria, which serves as their major nitrogen source, defaunation increases bacterial numbers in the rumen, thereby, increasing bacterial protein production (Viera, 1986), thereby suggesting that ciliated protozoa have a negative effect with regard to microbial protein supply (Table 1).

Ciliated protozoa have been shown to have a positive contribution to the ruminal fermentation of feedlot cattle fed high grain diets (85 to 95% grain diet). Historically, the contribution of ciliated protozoa to ruminal fermentation in feedlot cattle has been considered to be not significant because grain diets presumably reduce or even eliminate protozoal population. It was believed that rumens of feedlot cattle are inhospitable to ciliated protozoa because of low pH, hypertonicity and faster passage rates compared to forage-fed cattle. However, studies have shown that feedlot cattle harbor a dynamic population of ciliated protozoa, characterized by increased volatility and decreased diversity, with a small proportion of cattle (10 to 15%) defaunated, although transiently (Towne et al. 1990). Because of the predatory behavior of ciliated protozoa, the bacterial density and activity are higher in defaunated rumens. This is evidenced by ruminal pH values in grain-fed cattle. The presence of ciliated protozoa prevents a sharp decline in post-prandial ruminal pH in grain fed cattle (Figure 1; Nagaraja et al., 1992; Viera et al., 1983), which is a beneficial effect. The effect on pH could be attributed to their ability to influence starch and lactate metabolism in the rumen, thereby affecting VFA and lactate concentrations (Nagaraja et al., 1992; Mendoza et al., 1993). Ciliated protozoa have an

inverse relationship to lactate concentration in the rumen because of lower production and faster utilization of lactate. Differences in VFA and lactate concentrations are attributed to rapid uptake of readily fermentable sugars and starch, thereby sequestering them from immediate bacterial fermentation and enhanced lactate clearance from the rumen. Therefore, ciliated protozoa have a moderating effect on ruminal fermentation, in a way, exerting a buffering effect by slowing the rate of starch fermentation in grain-fed cattle (Nagaraja et al., 1992).

Another interesting aspect of the predatory ciliated protozoa of the rumen is their effects on bacterial pathogen survival and subsequent shedding in the feces (Stanford et al., 2010). There is also evidence that ciliated protozoa enhance virulence of pathogens, such as *Salmonella,* that leave the rumen (Rasmussen et al., 2005). The potential implication of virulence enhancement of pathogens by ruminal protozoa is not known.

Ruminal Fungi. Although flagellated zoospores, which are reproductive structures of fungi, were known even in 1900s, their identity as fungi was first described in 1975 by Colin Orpin (Orpin, 1975). Fungi that inhabit the rumen and contribute to ruminal digestion are anaerobic and form flagellated zoospores. The fungal contribution to ruminal fermentation is evidenced by the observation that selective elimination of ruminal fungi by chemical treatment resulted in decreased dry mater digestibility and feed intake in sheep (Gordon and Phillips, 1993). In the past, identification and description of fungi have been largely based on morphological features. Anaerobic fungi in the rumen belong to the family *Neocallimastigaceae* in the phylum Neocallimastogomycota, and so far nine genera and 20 species have been described (Edwards et al., 2017). The 18S rRNA in fungi, which corresponds to 16S rRNA in bacteria and archaea, has been used as a phylogenetic classification and quantification loci of anaerobic fungi. However, the 18S rRNA gene sequences are not variable enough to differentiate between all anaerobic fungi. Therefore, another region, called internal transcribed spacer 1 (ITS1) is used most extensively for differentiating genera and species of anaerobic fungi (Edwards et al., 2017). Based on ITS1 region sequence analyses, many other uncultivated fungal species exist (Edwards et al., 2017; Paul et al., 2018). Because of two-stage life cycle of anaerobic fungi (vegetative and zoospore stages), the quantification by microscopic enumeration or by culture methods is not meaningful. Based on chitin measurement, a macromolecule present in the fungal cell wall, and rRNA transcript, it is estimated that fungi account for 10 to 20% of the microbial mass in the rumen (Elekwachi et al., 2017).

Anaerobic fungi are the most active and effective fibrolytic organisms because of their combined mechanical (ability to penetrate plant structures) and enzymatic activities. The physical disruption of plant structures caused by fungal rhizoids increases plant surface area for colonization by bacteria. The efficient and extensive set of enzymes elaborated by anaerobic fungi contribute to their potent fibrolytic activity (Solomon et al., 2016). Also, fungi have a syntrophic association with methanogens, which contributes to increased fiber degradation. The interaction is mainly because of interspecies hydrogen transfer leading to methane production and efficient regeneration of oxidized cofactors and physical association of methanogens to fungal rhizoids and sporangia. Other than the studies on fibrolytic activities, not much is known of other fermentative activities of

fungi. A few studies have shown the benefit of feeding ruminal fungi as probiotics (Saxena et al. 2010). Among anaerobic fungi, ruminal fungi have been the most extensively studied, but in recent years, there is increased focus and research on their potential biotechnological applications (Gruninger et al., 2014).

Ruminal Viruses. Viruses are present in all microbial ecosystems and have been shown to be a driving factor in the evolution and stability of microbial communities. They play important roles in controlling the numbers and composition of microbes in an ecosystem by lysing susceptible microbes, selecting phage-resistant microbes, and facilitating horizontal gene transfer, a process called transduction. Bacterial viruses or bacteriophages, and possibly archaeal phages, are present in the rumen in high density. Phages can influence bacterial community composition and function because of their ability to lyse bacteria (lytic phages) or by getting incorporated into the chromosome (prophage) to alter bacterial function (lysogenic or temperate phages). Initial studies on bacteriophages were merely electron microscopic images and morphological description of bacteriophages (Klieve and Bauchop, 1988). A few of the lytic phages that have been isolated and somewhat characterized were specific to the genera *Sarcina* and *Streptococcus* (Adams et al. 1966). The number of bacteriophages has been estimated to be at 10^7 to 10^9 particles per ml of ruminal fluid (Swain et al., 1996).

Rumen viral community has also been investigated by metagenomics analysis (Anderson et al., 2017; Berg Miller et al. 2012). Viral genome analysis identified 2,243 viral populations and of those only 118 (5.3%) had significant similarity to known viruses. Based on taxonomic affiliations, 108 were identified as prokaryotic (bacteria) and 10 as eukaryotic phages (protozoa and fungi). Interestingly, none of the viral populations were characterized as archaeal (methanogens), perhaps because of low abundance of methane bacteria in the rumen and the poor representation of archaeal viruses in the database (Anderson et al. 2017). Prophages (lysogenic) were two-fold higher than the lytic phages with the most bacteriophages associated with the two dominant phyla in the rumen, Firmicutes (Gram positive bacteria) and Proteobacteria (Gram negative aerobic bacteria). The analysis identified a dynamic viral population but contained 14 ubiquitous viral populations suggesting the presence of a core rumen virome, in addition to novel viruses. Analysis of virally encoded auxillary metabolic genes indicates ruminal viruses have genes that code for glycosidic hydrolases, which could potentially contribute to fermentation of complex carbohydrates.

Because phages are pathogens to microbes, they have the potential to significantly alter ruminal function, which in turn could have negative or positive implications on feed utilization. Phages have been suggested as a possible mitigation strategy (bacteriophage therapy) to prevent acidosis (lyse *Streptococcus bovis*) reduce liver abscesses (lyse Fusobacterium *necrophorum*), inhibit methane production (lyse *Methanobrevibacter*). In the rumen, horizontal gene transfer has been implicated the dissemination of antimicrobial resistance genes (Toomey et al., 2009). Although there is no direct evidence phage encoded microbial activity, there are evidences of a transfer of a fungal gene that encodes for glycoside hydrolase into bacteria (Garcia-Valive et al., 2000) and bacterial genes encoding for plant cell wall polysaccharide degradation from bacteria into

ciliated protozoa (Ricard et al. 2006).

The molecular analyses of ruminal microbiota have generated considerable information on the community composition and has been widely used to understand factors affecting and changes associated with ruminal function and dysfunction. For the most part, rumen microbiome research is currently descriptive but is gradually moving to mechanistic, so that the knowledge can be translated into functional analysis and manipulations or interventions into ruminal fermentation to make it more efficient and the host more productive. The application of the molecular techniques and generation of new data on a topic of interest (methanogenesis) to ruminant nutritionists is presented below.

Methane Production in the Rumen: Significance and Mitigation Strategies

The fermentation of feeds by ruminal microbes produces hydrogen, which is used in several hydrogen-sink reactions, of which, methane production by archaeal population is the major route in the rumen. Methane is a waste product, hence, it is expelled into the environment, which results in the loss of energy to the animal and a source of greenhouse gas to the environment. Methane, as a greenhouse gas, is a major contributor, next only to CO_2, of global warming. Methane is more potent than CO_2 and estimated to account for 14% of total global greenhouse gas emissions. About 25% of the anthropogenic methane emissions are due to gut fermentations in livestock, particularly ruminants.

Although there is no relationship between methanogen abundance in the rumen to production efficiency of the animal, the species composition of methanogenic population is different between efficient and inefficient cattle (Zhou et al. 2009). In a study that used metagenomics analysis, a significantly higher abundance of *Methanobrevibacter* was detected in the rumen of high-methane producing steers compared to low-methane producers (Wallace et al., 2015). Interestingly, a couple of studies in sheep have noted differences in rumen microbiome beyond methanogens in relation to low- or high-methane producers (Kittelmann e al., 2014; Kamke et al., 2016; Wallace et al., 2015). Two bacterial genera, *Sharpea* and *Kandleria* (Kumar et al., 2018) were associated with low methane production. A metagenomic and metatranscriptomic study conducted by Kamke et al. (2016) confirmed the relative abundance of *Sharpea* was greater in low-methane producing sheep compared to high methane producing sheep. Not much is known about these two bacterial genera, except they are anaerobic and produce predominantly D-lactic acid from sugars. Not surprisingly, another organism that is significantly enriched in low methane producers is *Megasphaera elsdenii,* a major lactic acid-fermenting bacterium in the rumen (Kamke et al., 2016; Shabat et al., 2016). Thus, methanogenesis not only is related to methanogens but also other components of the microbiome, particularly lactic acid producers and fermenters. It is possible that lactic acid pathway (production and fermentation) may be central to the production of VFA as an alternative sink to methanogenesis (Mizrahi and Jami, 2018).

Ruminal methanogenesis results in the loss of energy (from 2 to 15% of digestible energy). Therefore, for a number of years, a major focus of researchers has been to develop an effective strategy to inhibit methane production in the rumen. The strategies that have been investigated can be broadly categorized to intervene at the following three

stages of methane production (Figure 2):

1. Inhibit or reduce production of major precursors of methane production (H_2 and formic acid);
2. Divert hydrogen to alternate hydrogen-sink reactions in the rumen; and
3. Eliminate or reduce methanogens in the rumen.

Because methane is the major scavenger of hydrogen in the rumen, methane inhibition results in hydrogen accumulation. It is generally assumed that hydrogen accumulation will inhibit re-oxidation of reduced cofactors like NADH and adversely affect the microbial fermentation. Therefore, strategies to mitigate methanogens should consider alternatives to sink hydrogen in the fermentation process (Wright and Klive, 2011). However, no negative effects of methane inhibition have been shown possibly because none of the methods tested inhibit 100% of methane production. Even an effective compound like bromochloromethane (BCM), which reduces methane production by about 80%, had no negative effective effects on feed intake and digestibility in goats (MItsumori et al., 2012). Although several inhibitors of methane production were effective in in vitro studies, they were reported to be ineffective in in vivo studies.

A promising compound appears to be 3-nitroxy propanol (**3-NOP**), an analog of the Coenzyme M that inhibits methyl coenzyme M reductase, which is present in all methanogens and is the terminal step in methanogenesis (Ermler et al., 1993). Several studies have shown that including 3-NOP in diets of dairy cows (Hristov et al., 2015) and beef cattle (Vyas et al., 2016) decreased methane emissions (up to 60%) with no negative effect on ruminal fermentation and animal productivity. Furthermore, inclusion of monensin in the diet had no significant interaction with the effects of 3-NOP (Vyas et al., 2018).

Conclusions

Rumen is inhabited by a dense population of microbes, which include members of all three domains of life: Bacteria, Archaea (methanogens) and Eukarya (fungi and protozoa), as well as viruses. The fermentative activities of these microbes convert complex organic feedstuffs into energy and protein, which are then used by the host for growth and production. Molecular methods to analyze bacterial community composition have identified a number of novel bacterial genera and species, which have not been cultured, therefore, nothing is known about their role in ruminal fermentation. Anaerobic fungi are the most active and effective fibrolytic organisms because of their combined mechanical (ability to penetrate plant structures) and enzymatic activities. Although ciliated protozoa contribute to digestibility of feeds and VFA production, their overall role in ruminal fermentation and contribution to the host nutrition is still an area of considerable debate and controversy. Rumen viral community analysis has identified a number of viral types and of those a small population have a significant similarity to known viruses. Viruses may be the driving factor in the evolution and stability of microbes in the rumen. Before the advent of molecular techniques, the understanding of the ruminal microbes and their contribution to the host nutrition was based on classical culture methods. In

recent years, there is explosive growth on the culture-independent methods, which have provided identity and quantity of microbes and have vastly expanded our understanding of the community composition. These studies are providing answers to who is there, and how many, but provide limited information on what are they doing. Cultivation and functional characterization of species and strains of microbes identified by molecular methods remain a major challenge to rumen microbiologists. An increased functional understanding of the microbiome of the rumen as well as that of the hindgut of ruminants is essential to develop novel approaches to manipulate to improve food animal production.

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Table 1. Effects of defaunation on ruminal microbial population and ruminal fermentation

Item	Ciliated protozoa	
	Present	Absent
Microbial population (per ml or g)		
Bacteria (70% roughage = 30% grain diet)[a]	0.7 - 3.1 x 10^9	5.2 - 10.5 x 10^9
Bacteria (15% rougahge + 85% grain diet)[b]	28 x 10^9	130 x 10^9
Fungi (no. of zoospores)[c]	0.3 x 10^4	0.3 x 10^4
Fermentation products[b]		
Ruminal pH	6.30	5.70
VFA concentration, mM	101.0	80.0
Acetate, mM	54.1	59.5
Propionate, mM	15.2	28.7
Butyrate, mM	8.9	8.2
Lactate, mM	0.07	0.30
Ammonia[c], mg/dl		
Before feeding	8.5	6.0
After feeding	7.5	0. 4
Carbohydrate fermentation		
Fiber digestion (% of intake)[d]	83	77
Starch digestion (% of intake)[e]	84.2	93.7
Nitrogen metabolism		
Dietary N degradability, %	68.7	61.0
Microbial N synthesis, g/100 g ruminal organic matter digestibility	3.56	5.03
Non-ammonia N flow, g/g N intake	0.93	1.09
Non-ammonia N flow, g/100 g OM intake	2.43	2.85

[a]Nuzback et al. (1983).

[b]Nagaraja et al. (1992).

[c] Males and Purser (1970).

[d] Ushida et al. (1990).

[e]Mendoza et al. (1993).

[f] Veira, D.M. (1986).

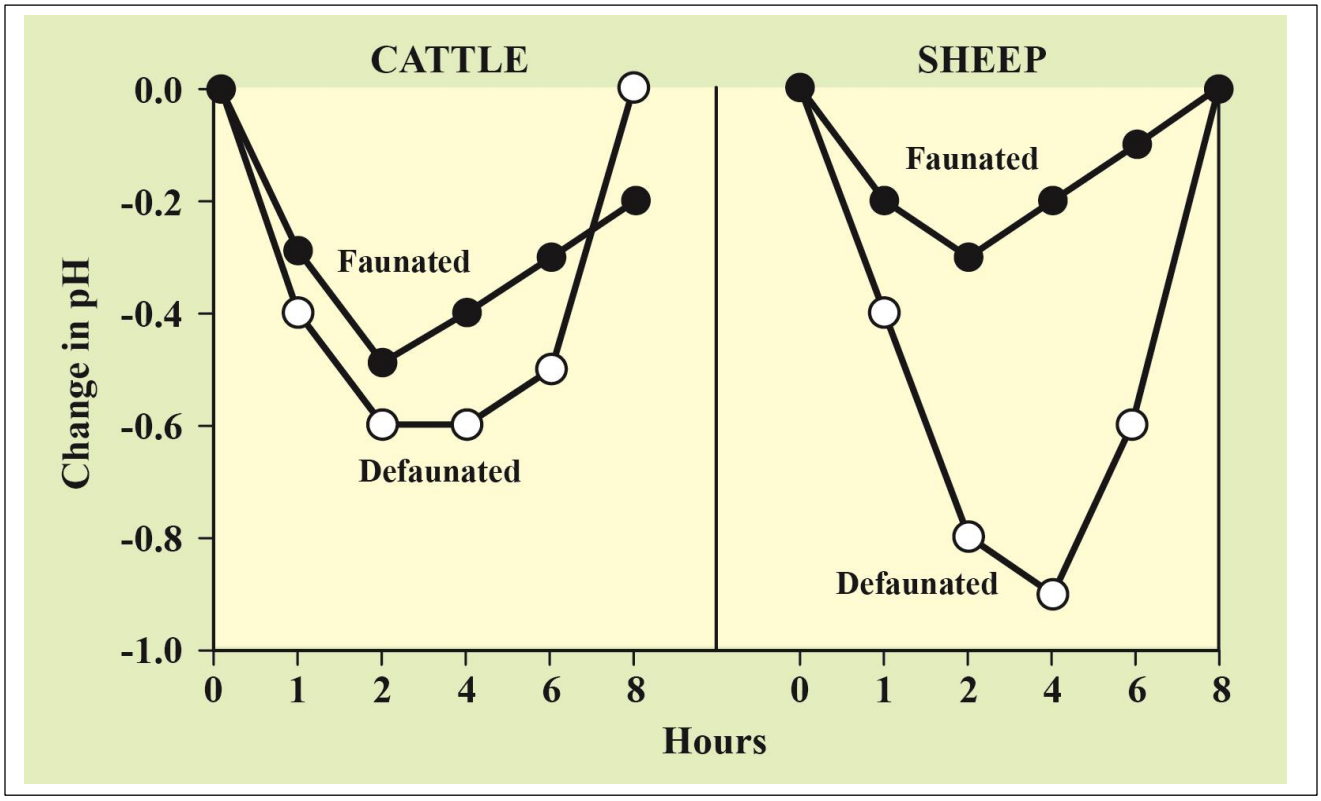

Figure 1. Post-prandial drop in ruminal pH in the presence (faunated) and absence (defaunated) of ciliated protozoa in cattle (Nagaraja et al., 1992) and sheep (Viera et al., 1986) fed high-grain diets.

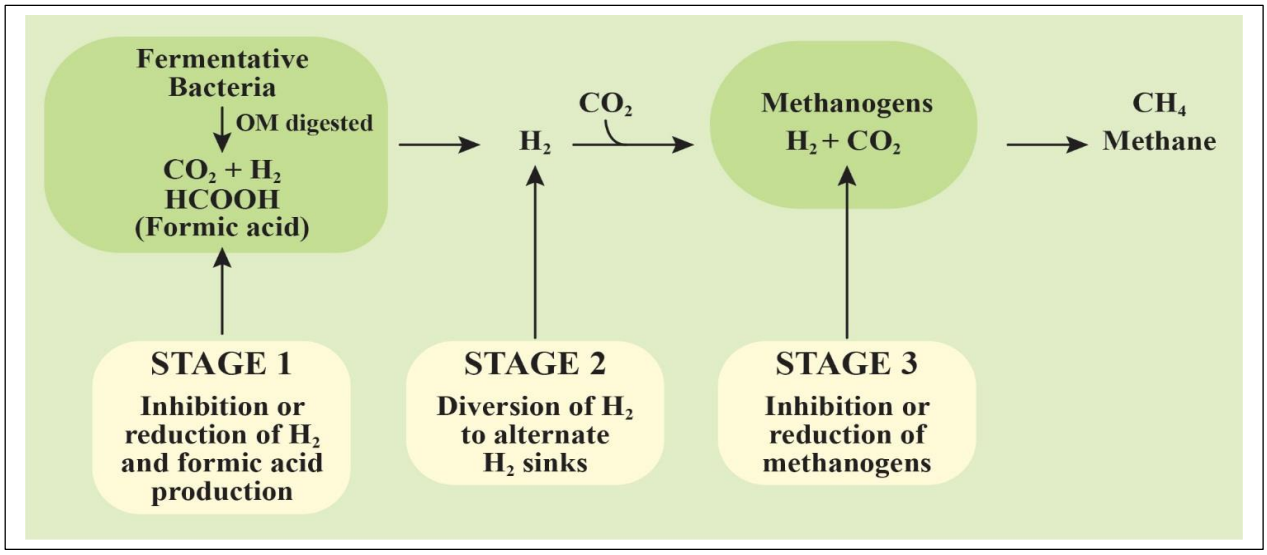

Figure 2. Stages in ruminal methanogenesis for intervention to inhibit methane production.

SESSION NOTES