Mining the rumen for fibrolytic feed enzymes

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Implications

- Demand for meat and milk is predicted to double by 2050, and meeting this increased demand represents a "grand challenge for humanity."
- Sustainable production practices for ruminants will require more efficient utilization of feed, with a greater emphasis on the use of fibrous feedstuffs.
- Fibrolytic enzyme cocktails have the potential to improve the nutritional value of low quality forages, such as straw, and improve overall feed efficiency in ruminants.
- Available commercial fibrolytic enzymes are not specifically developed for use in ruminant livestock and have not consistently improved ruminal fiber digestion.
- "-Omics" including, metagenomics and metatranscriptomics, have improved our understanding of rumen microbes and the enzymes involved in deconstruction of plant cell walls.
- A better understanding of the enzymes that limit plant cell wall deconstruction in the rumen could lead to more effective fibrolytic enzyme additives for ruminants.

Key words: fibrolytic enzymes, forages, metagenomics, metatranscriptomics, rumen

Introduction

With increasing affluence in emerging economies, the global demand for adequate and reliable supplies of meat and milk is expected to double by 2050 (Alexandratos and Bruinsma, 2012). With increased demand for animal products, farmers are faced with multiple socio–economic and environmental challenges, including: increased manure production and greenhouse gas emissions (**GHG**); livestock vs. human competition for cereal grains, and pressure to discontinue the use of antibiotics and growth promoters. Enhancing food security and sustainability poses a "grand challenge for humanity." Sustainable production practices for animal agriculture will require more efficient utilization of feeds and the introduction of new feed options.

Ruminants are unique in their ability to convert lignocellulosic biomass (i.e., grasses and crop residues) into high quality meat and milk protein for humans. The rumen microbiome is considered to be the most efficient microbial system at degrading lignocellulosic biomass (Flint et al., 2008), a fact that has attracted great interest in mining enzymes from this environment for use in the feed and food industry, cellulosic biofuel, and other industrial processes (Selinger et al., 1996; Hess et al., 2011; Wang et al., 2013).

This review will focus on current methodologies and strategies being used to identify rumen fibrolytic enzymes and develop effective ruminant fibrolytic feed enzyme cocktails. The complexity of plant cell walls and the enzymes involved in their digestion is also described to provide insight into future research targets.

Uniqueness of the Rumen

The rumen microbiome is exceedingly complex, being comprised of bacteria (up to 10^{11} cells/mL), protozoa (10^4–10^6 cells/mL), fungi (10^3–10^6 zoospore/mL), methanogens (10^6 cells/mL), and bacteriophages (10^7–10^{10} particles/mL) (Morgavi et al., 2013). These symbionts have evolved with the host for millions of years under conditions of anaerobiosis, high dilution rates, high cell densities and protozoa predation (Selinger et al., 1996). This selective pressure has accelerated the evolution of a microbiota toward a community that is highly specialized in degrading lignocellulosic biomass. The host is dependent on an array of enzymes produced by the microbial community to convert complex fibrous substrates into volatile fatty acids and microbial protein that are utilized by the ruminant for maintenance, growth, and lactation (Selinger et al., 1996; Morgavi et al., 2013). Even in light of this highly evolved environment, less than 50% of carbohydrates in low quality forages, such as straw, are digestible and useable by the host. Passage of lignocellulosic biomass from the rumen and restricted access of fibrolytic enzymes to targeted substrates are thought to limit the extent of plant cell wall degradation in the rumen (Weimer, 1996). Understanding the limiting steps and the complete mechanism of plant cell wall degradation by rumen microbes is essential for developing strategies to improve forage utilization in ruminants.

Past culture studies suggested that fibrolytic activity in the rumen was mainly driven by four bacteria, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens*, and to a lesser extent, by the rumen fungi (Krause et al., 2003; Morgavi et al., 2013). The adoption of cultivation-independent metagenomics and metatranscripitomics techniques has greatly enhanced the scientific communities' understanding of the rumen microbial ecosystem as well as mechanisms of plant cell wall digestion. It is estimated that less than 15% of rumen bacteria have been cultured in the laboratory (Morgavi et al., 2013). These cultivation-independent studies suggest that many of the microbes and lignocellulose-degrading enzymes participating in the ruminal digestion of plant cell walls remain uncharacterized (Hess et al., 2011). The Global Rumen Census (http://www.globalrumencensus.org.nz/home.html) determined that the composition of the rumen and camelid foregut microbial community (i.e., 742 samples from 32 animal species across 35

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countries) was influenced by diet, host species, and geography (Henderson et al., 2015). *Prevotella*, *Butyrivibrio*, and *Ruminococcus* and the unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales* were the most abundant bacterial groups in the rumen (67.1% of all bacterial sequence data) and formed the "core bacterial microbiome." Unclassified *Bacteroidales*, *Clostridiales, Ruminococcaceae*, and *Fibrobacter* were more abundant in cattle-fed forages compared with concentrate. This study and similar efforts such as the Hungate 1000 (http://www.hungate1000.org.nz/) and Rumenomics projects (http://www.ruminomics.eu/) continue to enhance our understanding of the rumen microbial ecosystem and its role in fiber digestion.

There is a growing appreciation for the important role that rumen fungi have in fiber degradation (Gruninger et al., 2014b). Anaerobic fungi physically disrupt the plant cell wall through invasive rhizoidal growth and the associated production of a range of powerful polysaccharide-degrading enzymes. The physical disruption and enzymatic activity of both fungal and bacterial enzymes act synergistically to degrade the most recalcitrant lignocellulosic material (Gruninger et al., 2014b; Fliegerova et al., 2015). Limited information exists on the role of protozoa in fiber degradation (Selinger et al., 1996; Devillard et al., 2003), but functional protozoal glycosyl hydrolases have been identified in the rumen. Findley et al. (2011) identified four novel glycoside hydrolases genes with two of these being successfully expressed and biochemically characterized. Interestingly, the xyloglucanse and arabinoxylanase they identified had higher specific activity then similar enzymes from rumen fungi.

Plant Cell Wall Structure

To better understand fiber digestion in the rumen and the enzymes involved, it is essential to recognize the complexity of the structural polysaccharides that make up the plant cell wall. Plant cell walls have diverse and critical functions including: providing structural integrity while maintaining the flexibility required for plant growth and enabling responses to environmental threats such as physical and microbial damage. There are two types of plant cell; primary and secondary walls. The primary wall is constructed first and surrounds all dividing and growing plant cells. The secondary cell wall surrounds plant cells that differentiate and undertake specialized functions (i.e., wood, xylem, and phloem cells). Secondary cell walls have polysaccharide compositions that differ from primary walls and are often lignified. The plant cell wall, including those in crop residues and forages, are almost entirely composed of cellulose, hemicellulose, pectin, and lignin (Fig. 1).

Cellulose is a linear polymer of glucose connected via ß-1, 4- linkages that form a flat, linear chain. Adjacent cellulose chains interact via hydrogen bonding and hydrophobic interactions. The hydrophobic interactions formed between the flat surfaces of the pyranose rings hold the chains together to yield crystalline microfibrils (Albersheim et al., 2011). Unlike cellulose, the backbone of hemicellulose is composed of a variety of sugars (i.e., glucose, xylose, and mannose) with a number of different types of branches. The branching and variable linkages between the sugars preclude hemicellulose from assuming the crystalline properties of cellulose. Pectin is a highly complex polysaccharide with α-1,4-linked galacturonic acids or α-1,2-rhamnopyranosyl residues as its backbone with further methyl-esterified or substituted acetyl groups. Finally, lignin is a complex polymer of aromatic alcohols that fills the spaces in the cell wall between cellulose, hemicellulose, and pectin. In effect, lignin acts as a physical seal around the cellulose and hemicellulose and impedes access of microbes and enzymes to these polymers. As a result, the linkages between lignin and hemicellulose are frequently viewed as the primary limiting factor of ruminal plant cell wall digestion.

Secondary plant cell walls have a large amount of xylan composed of ß -1,4-linked xylose residues. The xylan backbone has additional side groups that vary depending on plant species and tissue type. The frequency and type of branching, as well as the three-dimensional orientation of these branches, affect the ability of the xylan to hydrogen bond to cellulose or other regions of hemicellulose. In dicot plants such as alfalfa and soybean, the glucuronoxylan backbone of hemicellulose consists of xylose residues that are often branched with α-glucuronic acid or 4-methyl glucuronic acid and often acetylated. In contrast, monocot plant species such as barley and corn have a glucuronoarabinoxylan backbone that contains a large number of arabinose and methyl glucuronic acid side chains. Some of the arabinose branches are further ester linked to ferulic acid, or other hydroxycinammic acids, which connect the hemicellulose to lignin. Ferulic acid groups

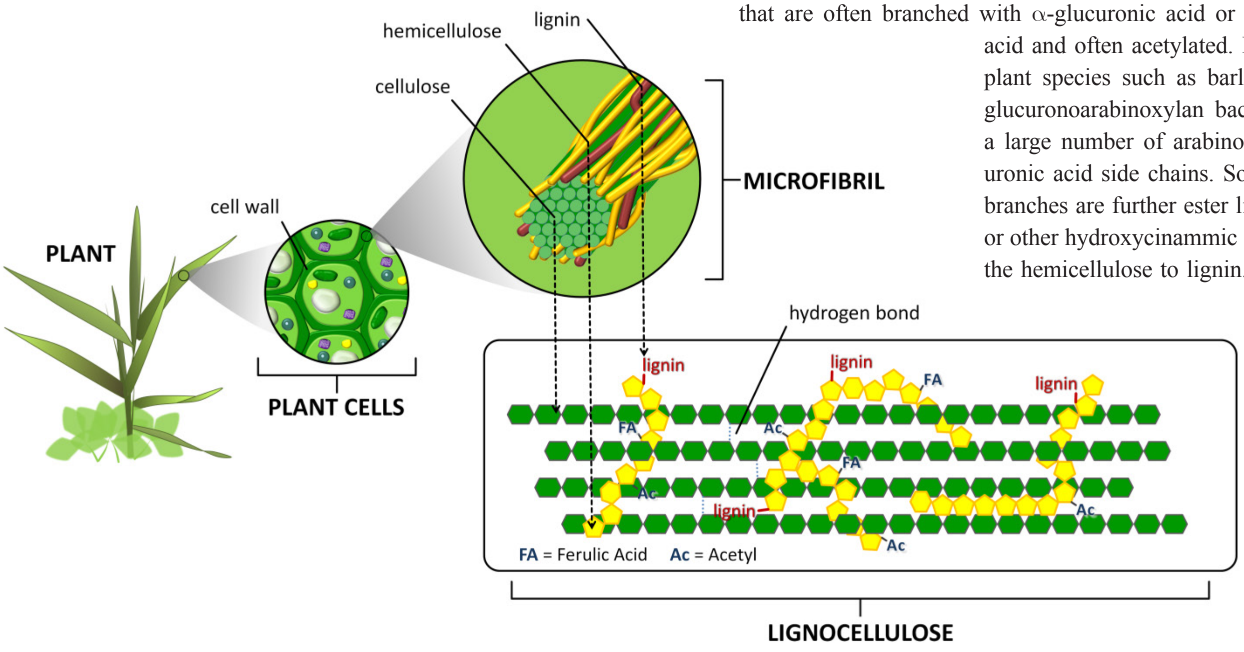

Figure 1. A simplified structure of insoluble plant fiber showing the crystalline cellulose in green and hemicellulose in yellow and its linkages with lignin, acetyl (Ac), and ferulic acid (FA).

can also dimerize and cross link xylan residues in adjacent hemicellulose backbone chains (Fig. 1) (York and O'Neill, 2008).

Enzymatic Deconstruction of the Plant Cell Wall

An array of enzymes is required to degrade lignocellulosic biomass owing to its heterogeneity in composition and structure. Efficient breakdown of cellulose requires the synergistic action of three classes of cellulolytic enzymes: endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). The variable structure of hemicellulose makes degradation of this polymer far more complex than cellulose, requiring enzymes capable of depolymerizing the hemicellulose backbone [endoxylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37), and glucuronoxylan hydrolases (EC 3.2.1.136)] as well as the side chains on the xylan backbone [α-L-arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterases (EC 3.1.1.72), feruloyl esterases (EC 3.1.1.73), and α-glucuronidases (EC 3.2.1.39)]. Many microorganisms, including fibrolytic rumen microbes, have evolved supramolecular complexes, termed "cellulosomes" and multifunctional enzymes that can hydrolyze a diverse range of fibrous substrates (Khandeparker and Numan, 2008).

Methods of Mining the Rumen Microbiome—Transcriptomics/Metatranscriptomics

The complexity of the rumen microbiota makes "-omics" based approaches, such as metagenomics and metatranscriptomics, ideal to study the phylogeny and function of this community (See sidebar). Most studies of the cattle rumen microbiome have focused on amplicon-based methods to describe how the types of microbes in the rumen differ with diet or host type. Amplicon-based metagenomic studies sequence regions of marker genes such as the 16sRNA, mcrA, 18sRNA, or ITS-1 that can be used to describe the bacterial, archaeal, protozoal, and fungal populations, respectively. These studies provide information on the composition of the microbial community, but little information about its function, particularly as it relates to the decomposition of plant cell walls within the rumen. Recent advances in sequencing andbioinformatics, along with a decrease in sequencing costs, have allowed researchers to perform shotgun metagenomic sequencing in which all genes within a rumen sample have the potential to be identified. Such an approach provides not only information on what organisms are present, but also information regarding the metabolic potential and function of the entire rumen microbial community.

To date, many of the "-omics" based studies have focused on characterizing the lignocellulose-degrading activity of the bacterial members of the rumen microbiome (Hess et al., 2011; Dai et al., 2015). The underlying goal of these studies is to uncover new ruminal fiber-degrading enzymes to expand a large database of "Carbohydrate Active enZymes" or CAZymes involved in the saccharification of lignocellulose and other carbohydrates. All of these studies discovered a large number of CAZymes, with many exhibiting low homology to previously described genes that code for proteins involved in plant cell wall degradation. This low level of sequence homology suggests that the encoded proteins may have unique biochemical properties.

While meta-genomic studies have focused on the rumen bacterial populations, it is well established that rumen fungi play a key role in the breakdown of lignocellulose (Gruninger et al., 2014b). A metatranscriptomic study of the fungal and protozoal communities in the muskoxen rumen found that CAZymes profiled in rumen solids differed substantially from those found using metagenomics (Qi et al., 2011). This finding was further corroborated by a similar study examining the metatranscriptome of the solid-associated microbial community in two Holstein cows (Dai et al., 2015). Metagenomic approaches tend to be biased toward the most numerically abundant genes harbored by the most abundant microbial species (Qi et al., 2011). However, the importance of a gene in plant cell wall degradation may not be necessarily reflected by its abundance. In contrast, metatranscriptome profiling provides a snapshot of the composition and relative abundance of actively transcribed genes (Qi et al., 2011; Dai et al., 2015). It is thus not surprising that the results of rumen metatranscriptomic studies are providing additional insight into the functional capacity of the rumen. By comparing metatranscriptome profiles from the rumen under different conditions, it is possible to derive functional information about how the rumen microbial community shifts and adapts to changes in the rumen environment.

Recent efforts have vastly improved our understanding of lignocellulose degradation in the rumen. The genome sequences of a number of key fiber-degrading rumen microbes, including fibrolytic bacteria (*Ruminococcus albus*, *R. flavefaciens*, and *Fibrobacter succinogenes*) and anaerobic fungi (*Orpinomyces* sp. C1A and *Piromyces* sp. E2) are available (Youssef et al., 2013, Denman and McSweeney, 2015). Most sequences obtained from rumen metagenomic and metatranscriptomic sequencing display low levels of identity to characterized CAZymes (Hess et al., 2011, Qi et al., 2011). This indicates that there is significant opportunity to identify highly efficient, novel CAZymes that could be developed for use in livestock nutrition as well as other industrial processes.

Commercially Available Enzymes

Commercial feed enzymes for livestock are produced using optimized fermentation systems and both natural and genetically modified fungi (mostly *Trichoderma reesei*, *T. longibrachiatum*, *Aspergillus niger*, *A. oryzae*, and *Pichia pastoris*) or bacteria (*Bacillus subtilis, B. lentus*, *Lactobacillus acidophilus, L. plantarum*, *L. bulgaricus*, *Enterecoccus faecium* spp., *Escherichia coli*, and *Penicillium funiculosum*). These production organisms were engineered to overproduce the enzyme of interest and deliver 50 to100 g of the active protein per liter of fermentation broth. Enzyme products can be concentrated and purified to ensure that no live cells, genetically modified recombinant DNA, or undesirable fermentation residues enter the final product (Adeola and Cowieson, 2011; Meale et al., 2014). Most of the fibrolytic enzymes produced commercially were developed for non-feed applications including the food, pulp and paper, textile, fuel, and chemical industries (Bhat and Hazlewood 2001). These enzyme products primarily contain xylanase (endo-1,4-β-xylanase) and glucanase [endo-1–3(4)-β-glucanase] activities, but a variety of additional ancillary enzyme activities (amylase, protease, pectinase, mannanase, and esterase) can be present (Adeola and Cowieson, 2011). These activities do not necessarily match those required for saccharification of plant cell walls, which differs depending on the chemical composition, porosity, and arrangement of the cell wall structure. Enzyme mixtures were also not formulated for optimal activities under rumen conditions. Furthermore, the specificity between the enzyme and the substrate has often been overlooked, factors that all contribute to the highly variable results observed when enzymes are added to ruminant diets (Meale et al., 2014).

What the Meta-"omics" Are You Talking About?

One of the major challenges in microbiology it that most microbes in the rumen cannot be cultured in the laboratory. This problem makes studying most bacteria out of the realm of "traditional microbiological' approaches. The solution has been to examine microbial communities as a whole as opposed to single species. This recent approach has been termed "meta-omics." Metagenomics is the most advanced of these "-omic" technologies involving isolation of genomic DNA fragments from an environmental sample, sequencing it, and computationally identifying the microbes that the DNA came from and reconstructing the microbial community. A related technology called metatranscriptomics isolates all of the RNA transcripts being expressed in an environment at a particular point in time. Not only can this approach be used to identify the types of microbes present, but it also provides information on their metabolic function at that particular point in time. Genes that are actively being transcribed will be found at high levels and will encode for proteins that are involved in metabolic processes. Examining how these transcripts change overtime, or under different conditions, provides information about how microbial communities adapt to their environment. In addition to metagenomics and metatranscriptomics, other more recently developed "-omic" based approaches involve examining all of the proteins in an environment (metaproteomics) as well as the metabolites (metametabolomics). Combining and correlating all of the information about the genetic makeup, gene and protein expression, and metabolites that are present in an environment provides insight into who is present, what they are doing, and how they are doing it; a branch of biology referred to as "systems biology" (Fig. 2).

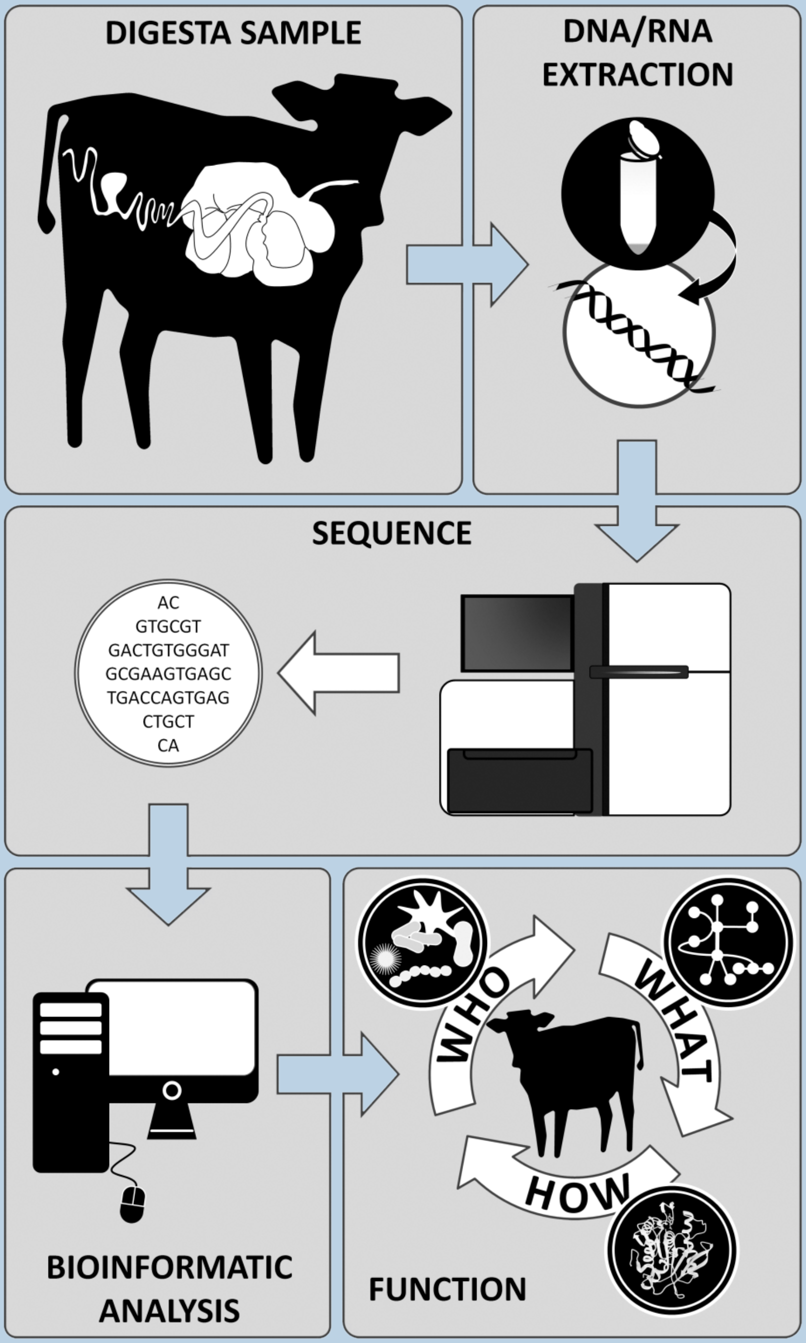

Figure 2. Steps used in "-omics" techniques as applied to the rumen microbiome.

Recent efforts using meta-omic technologies to examine the rumen have led to great strides in our understanding of rumen lignocellulose degradation. To date, information on the types of microbes and enzymes that are present in the rumen has not been used to formulate fibrolytic enzyme products that function under rumen conditions or act synergistically with the natural enzymes produced by the rumen microbial community. A successful enzyme product will undoubtedly have to focus on rate-limiting enzymes in the rumen such as ferulic acid esterases and enzymes involved in hemicellulose side chain removal. These enzymes are involved in the solubilization of lignin, and increasing the access of other enzymes to cellulose and hemicellulose.

Strategies for Discovering Microbes and Enzymes that Can Improve Rumen Digestion

In contrast to most domesticated ruminants (cattle, sheep, and goat), which are frequently fed grain or high quality forages, wild ruminants (i.e., bison, elk, deer, moose, and muskoxen; Fig. 3) and their gut microbes have co-evolved to utilize natural feedstuffs that are high in lignocellulose (low quality forages). The work examining the lignocellulose-degrading enzymes in the rumen of muskoxen demonstrates the potential for wild ruminants to be a source of potent fibrolytic enzymes. Recent studies have demonstrated that

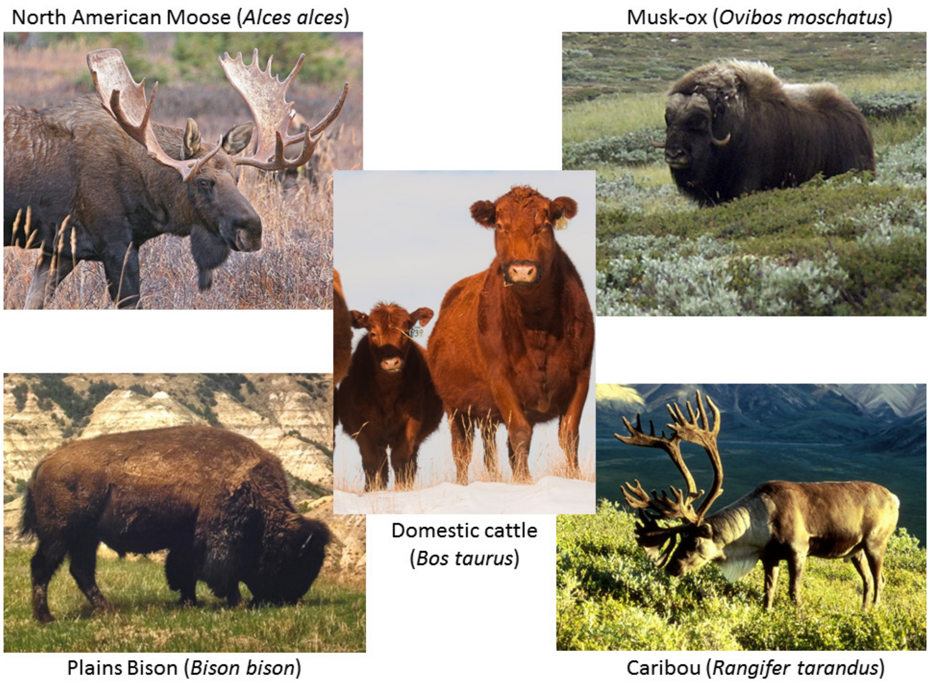

Figure 3. Example of ruminants used in the search for novel fibrolytic enzymes within the natural microbial ecosystem of the rumen.

although differences in microbial community compositions among ruminants are predominantly attributable to diet, the host also has some degree of influence on its composition (Gruninger et al., 2014a; Henderson et al., 2015).

Characterizing the microbial community from the rumen of cattle that are more effective at plant cell wall degradation to those that are less efficient may also help provide further insight into the key microbes and enzymes involved in the deconstruction of plant structural polysaccharides. With this concept in mind, recent studies have compared the microbial community of cattle with diverging residual feed intake (**RFI**; Guan et al., 2008; Carberry et al., 2012; McCann et al., 2014). The RFI is defined as the divergence in feed intake from a predicted intake based on rate of gain and body size. Inefficient cattle have a positive RFI, as they eat more than predicted, whereas cattle that have a negative RFI eat less than predicted and are thus more efficient. Results from these studies suggest that with high forage diets, the rumen microbiome of less efficient (positive RFI) cattle harbor more *Prevotella* (Carberry et al., 2012; McCann et al., 2014). Significantly higher concentrations of rumen butyrate and valerate were also detected in more efficient steers (Guan et al., 2008). These studies suggest that there may be a relationship between ruminal bacterial diversity and feed efficiency and that more in-depth studies of the ruminal microbial community of cattle that differ in their fiber digestion capacity is warranted.

Rumen transfaunation is one approach that has been used to introduce unique rumen microorganisms into animals. Transfaunation involves physically introducing microbial communities into the rumen of cattle and has been successfully used to clinically treat indigestion and to convert toxic compounds found in some plants to harmless or even beneficial compounds (DePeters and George, 2014). However, attempts to directly improve fiber digestibility through the addition of specific bacterial species has proven elusive. For example, introduction of the cellulolytic bacteria *Clostridium longisporum* ATCC 49440 or *C. herbivorans* ATCC 54408 into the bovine rumen was unsuccessful as neither bacterium persisted in the rumen for more than 72 h (Varel et al., 1995). Other studies that dosed fibrolytic bacteria into the rumen have also proven unsuccessful at enhancing fiber digestion as these bacteria also failed to persist (Attwood et al., 1988; Flint et al., 1989; Miyagi et al., 1995). Rumen fibrolytic bacteria reproduce and persist as members of a complex microbial community that remains largely undefined. Presumably, members of this complex microbiome work cooperatively, with some members cleaving specific bonds within the plant cell wall architecture, providing other members of the community with access to substrates (Krause et al., 2003). The ability to target and utilize specific substrates in the plant cell wall enable microbes in this complex community to coexist by exploiting niches and minimizing competition for the same resources. Success in establishing introduced bacteria into the rumen, as with *Synergistes jonesii*, which degrades the toxin, mimosin, appears to depend on the introduced bacterium occupying an unique, energetically favorable niche within the microbial community (Jones and Megarrity, 1986; Weimer, 1998). Such an unique niche may not be as readily identifiable, given the complexity of the microbial community involved in plant cell wall digestion.

There is also evidence that the rumen microbiome may also be host-specific, possibly raising barriers to the establishment of introduced bacteria across different hosts (Weimer et al., 2010). To examine the specificity of a cow's ruminal bacterial community, more than 95% of the rumen contents were swapped among cows fed the same diet, but differing in ruminal pH, VFA concentration, and bacterial communities. Bacterial communities reverted back to those of the original host within 14 to 62 d, and surprisingly, pH and VFA returned to original levels within 24 h after exchange of rumen contents (Weimer et al., 2010). Given these findings, one could envision that the successful establishment of a bacterial, protozoal, or fungal strain in the rumen would depend on its ability to occupy a niche as a result of producing rate-limiting and/or unique enzymes that overcome the initial limits of plant cell digestion.

Future Mining Targets

Ultimately, enhancing the nutritional value of low quality forages requires improving the accessibility of enzymes and microbes to both crystalline cellulose fibrils and hemicellulose. Lignin is not digested in the rumen and serves as a barrier that limits access to these digestible polymers. Through breaking the ester linkages between lignin and hemicellulose, fibrolytic enzymes can enhance degradation by gaining better access to core structure of the plant cell wall. Aerobic microbes that digest lignocellulose have evolved oxidative enzymes that hydrolyze lignin, but these enzymes do not function within the anaerobic environment of the rumen. The rumen contains a large number of esterases that target both acetylated xylan and hydroxycinnamic acid linkages within hemicellulose. The action of these enzymes removes side chains from the xylan back bone, enabling the action of endo- and exo-xylanases within the rumen. An increasing number of rumen meta-omic studies are shedding light on potentially missing and/or limiting enzyme activities within the rumen. It is now known that cellulases of the classes GH7 and GH12 are not present in the rumen but are found in aerobic environments (Riley et al., 2014, Dai et al., 2015). Furthermore, our work has shown that supplementing rumen enzyme mixtures with aerobic enzymes results in a synergistic release of glucose and xylose from barley straw and alfalfa hay (Badhan et al., 2014). The synergism between aerobic and anaerobic enzymes suggests that they may target unique sites within lignocellulose and thus do not compete for the same binding sites. Finally, a number of swollenin and expansin proteins have been found within the rumen (Qi et al., 2011). These proteins bind to the plant cell wall and physically alter lignocellulose by disrupting hydrogen-bonding networks, increasing the accessibility of cellulose to enzymes (Wang et al., 2011). Despite the lack of cellulolytic activity, the addition of a fungal swollenin to crystalline cellulose resulted in a synergistic increase in cellulase activity of an endoglucanase that lacked activity against crystalline cellulose (Wang et al., 2011).

Conclusion

The rumen microbiome has long been considered to be an excellent source of fibrolytic enzymes. The development and adoption of molecular biology approaches (i.e., metagenomics and metatranscripitomics) that are independent of cultivation-based methods has yielded considerable information about the microbes and carbohydrate active enzymes involved in ruminal lignocellulose degradation. Although efforts at characterizing these activities has begun, researchers have yet to develop an effective ruminant fibrolytic feed enzyme cocktail that functions not only under rumen conditions, but also acts synergistically with the natural enzymes present in the rumen. A better understanding of the rate limiting enzymes to fiber deconstruction in the rumen will definitely play an important role in this process.

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Dr. Gruninger completed his Ph.D. in biochemistry at the University of Lethbridge in 2009 in which he used biophysical and structural approaches to characterize the molecular basis of catalysis and substrate specificity of the enzymes responsible for phytate degradation in the rumen. This was followed by a post-doctoral fellowship from 2009–2012 at the University of British Columbia using structural biology to probe the determinants of antibiotic resistance to beta-lactam antibiotics in important human pathogens. Gruninger has been conducting research as a research associate at the Lethbridge Research Centre since 2012. The focus of his research is to apply a combination of "-omics" based techniques and protein biochemistry to better understand the biology of ruminant microbes, particularly the anaerobic fungi and their role in lignocellulose degradation in the rumen.

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