Current Knowledge of the Ruminal Fermentation System and What Can We Expect to Learn in the Future

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Introduction

The ruminal fermentation system is simply the fermentation of dietary organic matter **(OM)** in the rumen, resulting in usable end products (e.g., volatile fatty acids **(VFA)**, microbial protein, and long chain fatty acids **(LCFA)**) for the dairy cow. This fermentation system has the unique ability to degrade, convert, or alter over 85% of the cow's diet, which means the rumen compared to all of the body's organs exerts the most influence on milk production and composition. The function of the rumen and the microbial community housed within are, therefore, critical to optimizing the performance of the dairy cow. How the rumen is physically structured and fundamentally works is explained well in numerous reviews and books (Van Soest, 1982; Krehbiel, 2014) and will not be the focus of this paper. The focus of this paper will be on our current knowledge of the rumen microbial communities and potential opportunities to manipulate milk synthesis and composition.

Regulation of Milk and Milk Component Synthesis

Regulation of milk and milk component synthesis in the mammary gland is related to 3 areas: milk volume, milk protein yield and composition, and milk fat yield and composition. Milk volume is under osmotic regulation with lactose as the major osmolyte that regulates the amount of water drawn into the aveoli of the mammary gland (Akers, 2002). As a result, those diets that promote the major precursor for gluconeogenesis, propionate, have the ability to increase lactose synthesis, and in turn, milk volume, especially in early lactation (McCarthy et al., 2013).

Milk protein yield is controlled primarily by the metabolizable energy supply from the diet via insulin, IGF-1, and other energy signaling pathways, and by the substrate supply of essential amino acids **(EAA)** in the blood (Bionaz, et al., 2012). Changing energy content of diets will change milk protein yield (Reynolds, et al., 1994), while changing energy balance through feed restriction and realimentation has been shown to change milk protein percentage, but not milk fat percentage (Gross, et al., 2011). Increased circulating blood insulin levels have been shown to increase milk protein yield (Winkelman and Overton, 2010).

The EAA profile of rumen-generated microbial protein **(RGMP)** is highly similar to the EAA profile of milk, and the intestinal digestibility of microbial protein is consistent and generally greater than sources of rumen undegradable protein **(RUP)** (Block, 2006). Depending on the microbial efficiency, RGMP can theoretically represent 50 to 79% of the total metabolizable protein **(MP)** needs of

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a high-producing dairy cow (Block, 2006). However, the variability of EAA composition in RGMP is quite high (Clark et al., 1992), which could affect predictions of MP EAA levels in the various software models in the market. The composition of the protein fractions in milk ,on the other hand, are minimally influenced by nutritional changes in the cow's diet (Hadrova, et al., 2007).

Milk fat yield and composition are both highly influenced by dietary factors (Bauman, et al., 2008). De novo synthesis of medium and short chain FA in the mammary gland is controlled by substrate supply and production of inhibitory isomers of conjugated linoleic acid **(CLA)**. The incorporation of LCFA into milk fat is also affected by the dietary supply as modified by ruminal biohydrogenation. Acidosis and subacute ruminal acidosis **(SARA)** has been intimately linked to incomplete biohydrogentation of LCFA in the rumen and the occurrence of milk fat depression **(MFD)** (Bauman et al., 2008; Khafipour et al., 2009). Therefore, manipulation strategies that change or stabilize the supplies of energy, AA, or LCFA coming from the rumen have an opportunity to change or stabilize milk yield and composition.

The Rumen Microbiome

Microbiome is the term used to describe the totality of all of the microorganisms residing in a particular environment (e.g., rumen). Historically, we have been trained that there are 3 distinct populations of microorganisms that make up the rumen microbiome: bacteria, protozoa, and fungi (Van Soest, 1982). However, with the advent of improved culture techniques and molecular biological techniques, such as 16S rRNA sequencing, we have come to recognize other distinct populations of microorganisms that exert influence on rumen function: archaea (i.e., methanogens) and bacteriophage (i.e., viruses).

Bacteria

The bacteria in the rumen are the most significant population of microorganism with 1010 CFU per g of rumen contents (Russell, 2002) and representing 60 to 85% of small subunit rRNA (Lin et al., 1997). Our understanding of the diversity of the bacterial species in the rumen has increased from those we can culture, approximately 200 recognized species, to those we cannot, > 3,500 operation taxonomic units (OTU), which is the species level designation when only DNA sequence data are available (Kim et al., 2011). Given this diversity, the bacterial population is normally discussed or grouped based on main substrate fermented: starch-degraders or amylolytic, fiber-degraders or cellulolytic, protein-degraders or proteolytic, fat-utilizers or lipolytic, etc.

Protozoa and fungi

The protozoa are divided into 2 types, flagellated and ciliated, and though low in number (103 to 106 per g of contents), physically they can amount for 50% of the cellular biomass (Dehority, 2003). The genetic diversity of protozoa is limited to < 50 OTU (Kim et al., 2011). Protozoa, because of their bacterial predation, are major players in N recycling, starch degradation, and fiber breakdown, as well as maintaining a symbiotic relationship with the methanogens (Firkins, 2012).

Fungi in the rumen are considered to be important in fiber degradation due to their extensive mycelial structures that invade plant tissues and their wide array of cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes (Liggenstoffer et al., 2010). However, they are present in small numbers (103 spores per g of contents) with limited genetic diversity (10 to 60 OTU) (Fouts et al., 2012).

Archaea (i.e., methanogens)

The methanogens were for many decades classified as members of the bacterial community, but the work of Woese (1987) redefined the evolutionary status of the methanogens to a new domain, Archaea. The methanogens scavenge H_2 in the rumen, allowing for more complete fermentation of substrates but at the cost of approximately of 5 to 7% of dietary gross energy **(GE)** through methane emissions, which makes cows targets for greenhouse gas **(GHG)** reduction strategies worldwide (Hristov et al., 2013). The Archaea are present at 107 cells per g of contents.

Bacteriophage (i.e., viruses)

Bacteriophage, often referred to as phage, are bacterial viruses that are involved in lysis of bacterial cells, as well as horizontal transfer of genetic material (Kleive et al., 1996). They occur at 10^9 to 10^{11} particles per mL of rumen fluid and are distinctly associated with the predominant bacterial populations in the rumen (Berg Miller et al., 2012).

Manipulation of the Rumen Microbiome

Historically, we have assumed that we have changed the microbial population dynamics in the rumen with a variety of feed additives. These shifts in populations were ascribed by the circumstantial evidence of changes in pH, VFA concentrations, microbial protein flow, and digestibility of different diet fractions (e.g., fiber, starch, protein, etc.). Assessment of actual changes in microbial populations was limited to those few organisms that were culturable (Krause et al., 2013). Over the last 30 years, the development of molecular biological techniques have given rise to the field of metagenomics, which allow for culture-independent analysis of the changes in the rumen microbial ecosystem (Krause et al., 2013). For a very good review of these techniques, please refer to Chaucheyras-Durand and Ossa (2014) and McCann et al. (2014). In the future, these techniques will continue to increase our understanding of the dietary and environmental impacts on the rumen microbiome, and how those impacts can be replicated to provide consistent and measurable performance responses in dairy cattle.

The idea of consistency leads to a discussion of responders and non-responders. Why do some cows respond to a feed additive or diet change while others do not? Genetic finger printing studies demonstrate how animal-to-animal variation controls the rumen microbial ecosystem. Work at the USDA Forage Center in Wisconsin by Weimer and colleagues illustrated clearly the impact of the cow when they performed near-total exchange (>95%) of rumen contents between 2 cows with very different ruminal pH, VFA concentrations, and bacterial community compositions **(BCC)** and followed the changes in BCC for the next 60+ days (Weimer et al., 2010b). Ruminal pH and VFA for both cows returned to pre-exchange levels within 24 hr. However, the BCC of both cows returned to original pre-exchange profiles in 14 and 61 days, respectively. These results show that the cow has tremendous control over ruminal pH and VFA content, even though the BCC is the source of VFA production.

If a cow can recover its original BCC from a perturbation, such as a one time, near total exchange of rumen contents, then how do cows and their rumen microbiome respond to perturbations from daily dietary changes or feed additives? In work examining BCC under MFD conditions, Weimer et al. (2010a) screened 18 cows to find clusters of the cows that demonstrated MFD with either rapidly

fermented starch **(RFS)** or monensin **(M)** addition, or the combination of both **(RFS/M)**. The researchers then compared the BCC of the liquid and particle associated bacterial populations within each cluster of cows to determine how the BCC shifted with each dietary treatment. Several interesting results were observed: 1) Cows within a cluster (i.e., having the same response in milkfat to a specific treatment) had different BCC, 2) The liquid and particle associated BCC were similar within individual cows, 3) Cows sensitive to RFS or M demonstrated large changes in BCC while cows sensitive to both RFS/M and non-responding cows showed small changes in BCC, and 4) For cows that demonstrated a MFD response to diet, the BCC did not return to its original structure with removal of monensin from the diet. Together, these results suggest that while the cow dictates its individual BCC, the responses to MFD inducing diets are directly associated with changes in BCC.

If the cow exerts such remarkable control over the rumen through passage rate (i.e., intake), buffering through saliva production, and rate and extent of absorption of VFA, then how similar is the rumen microbiome between cows consuming the same diet? Jami and Mizrahi (2012) compared the rumen bacterial populations across 16 cows fed the same diet consisting of 70% concentrate:30% forage. The researchers found that of 250 OTU identified, 32% were present in 90% of the cows and only 19% of the OTU were present in all of the cows. In terms of abundance (i.e., amount) of each OTU in individual samples, there was < 60% similarity across samples. These results point to low similarity in the rumen bacterial populations both in presence and level of the OTU, which may have an impact on how effective dietary changes are across a group of animals. On the other hand, there may be a core bacterial population that if properly defined and targeted could allow for more consistent responses to dietary changes.

Opportunities to Manipulate the Rumen Microbiome and Possibly Cow Response

Shifting dairy cattle diets to generate more propionate (i.e., propiogenic) versus acetate is often accomplished by increasing dietary starch content or starch fermentability, or by addition of monensin (McCarthy et al., 2013). However, the effect of these treatments on the rumen microbiome has been shown to be variable. Belanche et al. (2012) fed 11.7 vs. 30% starch diets to lactating Holstein cows, and found decreases in protozoa (-38%), fungi (-59%), and methanogens (-27%), while total bacteria did not change with high starch diets. However, known cellulolytic bacterial populations were unaffected by increasing dietary starch content. Working with lactating Holstein cows, Lettat et al. (2013) fed diets with 0, 50, or 100% of the dietary forage as corn silage **(CS)**, which linearly increased dietary starch from 17.0 to 30.0%, and found a 4-fold decrease in protozoa, a 2-fold increase in total bacteria, and a 1.5-fold increase in methanogens. And, while ruminal pH declined linearly with increasing CS in the diet, the known cellulolytic bacterial populations did not significantly change. Thoetkiattikul et al. (2013) fed crossbred dairy cows diets containing either 2, 10, or 21% starch and found a linear decrease in the genera of cellulolytic bacteria with increasing starch level. So, while increasing dietary starch can increase propionate available for gluconeogenesis, the rumen microbiome response across cows has not been defined such that a consistent response can be expected.

The most common benefit afforded to monensin is the inhibition of Gram-positive **(G+)** bacteria, which shifts the ruminal fermentation to greater propionate

concentration at the expense of acetate concentration (McGuffey et al., 2001). However, numerous studies demonstrate no effect on the acetate:propionate ratio (Oelker et al., 2009; Mathew et al., 2011; Reveneau et al., 2012), which has been attributed to rapid adaption of G+ bacteria (Weimer et al., 2008). As previously described, ruminal bacterial populations exhibit variable changes to monensin supplementation (Weimer et al., 2010a). Monensin supplementation does not change total protozoal levels in the rumen but does cause small variations in the population composition (Arakaki et al., 2000; Reveneau et al., 2012). Archaeal populations in the rumen show little change to monensin supplementation (Hook et al., 2009). Therefore, the effect of monensin on the rumen microbiome may be related to impacts on individual bacterial species rather than whole populations.

The variability of the nutrient composition of microbial flow from the rumen is well documented for both EAA profile of the RGMP (Clark et al., 1992; Martin et al., 1996) and the fatty acid profile (Or-Rashid et al., 2007). That variability may be related to the proportions of protozoa and bacteria flowing from the rumen, as well as proportion of liquid vs. solid-associated bacteria (Belanche et al., 2011). Firkins and colleagues at The Ohio State University have done extensive research on the recycling or selective retention of protozoal populations in the rumen, and how that will affect RGMP flow (Firkins et al., 2007). The presence of protozoa has been shown to increase the ratio of 2 major bacterial phyla, Firmicutes:Bacteroidetes, and increase ammonia-N levels in rumen contents (Ozutsumi et al., 2005). Treatments to alter or reduce EAA profile variation in RGMP should consider effects in both the liquid and solids fractions, since the liquid and solids-associated populations of both protozoa and bacteria have different EAA profiles and different flow rates from the rumen (Martin et al., 1996; Hook et al., 2012).

The manipulation of milk fat content and composition through alterations in the rumen microbiome is heavily challenged by the relationship between acidosis/SARA and biohydrogenation of LCFA. The definition of acute acidosis is the sudden and uncompensated drop in rumen pH to < 5.0 (Krause and Oetzel, 2006), while the definition of SARA is prolonged periods of moderately depressed ruminal pH (Krause and Oetzel, 2006; Plaizier et al., 2009). There is disagreement in the literature as to the pH threshold for SARA onset varying from 5.5 to 6.0 (Krause and Oetzel, 2006; Plaizier et al., 2009). While the ruminal fermentation conditions of SARA are similar across studies, the changes in the rumen microbiome vary widely, depending on the causative agent (Khafipour et al., 2009). Khafipour et al. (2009) demonstrated that grain-induced SARA causes significant increases in *S. bovis*, an amylolytic bacterium producing lactic acid, and concomitantly, *M. elsdenii*, a lactate-utilizing bacterium. When Khafipour et al. (2009) induced SARA with pelleted alfalfa, there were no changes in *S. bovis* or *M. elsdenii*, but *Prevotella* spp. increased significantly in relation to other known bacterial species. These differences in bacterial composition could drive how we work to prevent or treat SARA on a farm level. Grain-induced SARA is directly related to diet formulation; whereas, alfalfa pellet-induced SARA is related to both diet formulation and feeding management as particle size of dietary components is a key factor in the latter.

Based on the biohydrogenation theory of MFD (Bauman et al., 2008), dietary and feeding management factors that alter ruminal fermentation (e.g., elevated starch or grain, oil,

presence of monensin, or reduction in particle size) will result in altered ruminal FA metabolism due to changes in the rumen microbiome, increasing the flow of polyunsaturated FA **(PUFA)** through an alternative pathway of biohydrogenation. The SARA induction models described by Khafipour et al. (2009) fit with the biohydrogenation theory as *M. elsdenii*, elevated during grain-induced SARA, and *Prevotella* spp., elevated during alfalfa pellet-induced SARA, have been implicated in MFD (Palmonari et al., 2010; Jami et al., 2014).

More recently, Jami et al. (2014) was able to demonstrate a relationship between the ratio of 2 major bacterial phyla, Firmicutes:Bacteroidetes, in the rumen and daily milk fat yield. Across 15 cows fed the same diet, an increasing Firmicutes:Bacteroidetes ratio was positively correlated with milk fat yield (R^2 = 0.51). In humans and mice, an increased Firmicutes:Bacteroidetes ratio in the gut microbiome is associated with increased energy harvest and body fat tending towards obesity (Ley et al., 2006; Turnbaugh et al., 2006). Additionally in the work of Jami et al. (2014), among the 42 common core genera (i.e., those genera found in >50% of the cows sampled), *Prevotella*, found in the Bacteroidetes phylum, were strongly negatively correlated with milk fat yield (Pearson R = -0.69, P = $5x10^{-3}$). On the other hand, *Bifidobacterium* and *Lactobacillus*, both common probiotic genera, were positively correlated with milk fat yield. Weimer et al. (2010b) also found specific species of bacteria were associated with the responsiveness of individual cows to MFD inducing dietary treatments. Therefore, even with a limited core of bacterial species across cows, defining and targeting those bacterial species involved with specific performance responses may represent an opportunity to modulate milk composition in the future.

Aside from diet formulation and feeding management, the opportunities for controlling SARA and MFD by changing the rumen microbiome may rest with probiotics. Probiotics are, by definition, viable microorganisms or endproducts of their fermentation that when consumed in adequate amounts confer a health benefit on the host (FAO, 2001). In the dairy cattle industry, there are 2 general groups of probiotics, bacterial-based and fungal-based, which are termed, direct-fed microbials **(DFM)**.

Bacterial-based DFM normally contain a variety of species with wide ranging metabolic activities. There are limited demonstrations of changes in the rumen microbiome with bacterial-based DFM. Chiquette (2009) using culture-dependent techniques, demonstrated that *E. faecium* and *S. cerevisiae* **(ES)** fed under SARA conditions did not affect *R. flavefaciens*, *F. succinogenes*, *R. albus*, or *M. elsdenii* levels in lactating dairy cows. More recently, Chiquette et al. (2012) using a combination of ES and *P. bryantii* **(PB)** were able to demonstrate an increase in *R. flavefaciens*, but no effect on *F. succinogenes*, *R. albus*, or *M. elsdenii* levels in lactating dairy cows fed to induce SARA. The supplementation of PB alone had no effect on any measured bacterial populations.

Fungal-based DFM are either a live yeast or yeast culture. Live yeast are defined as active dry yeast products that must contain >15 billion live yeast cells/g (AAFCO, 2011). Yeast cultures **(YC)** are products from yeast fermentation that contain live yeast and fermentation by-products and are not dependent on live yeast for their physiological effects (AAFCO, 2011). There are numerous benefits to rumen function attributed to fungal-based DFM: 1) stimulate growth of beneficial microorganisms, 2) improved fiber digestion, 3) reduced lactate concentrations, 4) reduced O_2 concentrations, 5) improved starch utilization, and 6) moderation of ruminal pH.

The suggested mode of actions that elicit those benefits include: production of stimulatory AA, peptides, vitamins, and organic acids; out competing lactate-producing bacteria for available carbohydrates; and scavenging of O_2 by live yeast cells. There are numerous thorough reviews of yeast-based DFM available in the literature (Chaucheyras-Durand et al., 2012).

There are numerous studies that demonstrate changes in the rumen microbiome with yeast supplementation. Harrison et al. (1988) fed YC to ruminally fistulated Holstein cows, and using culture-dependent techniques, they found yeast supplementation to increase total anaerobic bacteria and cellulolytic bacteria compared to controls. Mathieu et al. (1996), using culture dependent techniques, also found an increase in total bacteria but a decrease in cellulolytic bacteria with supplementation of YC. Arakaki et al. (2000) examined the impact of YC on protozoa counts in ruminally fistulated steers and found that while the total protozoal counts did not change, the protozoal species composition changed with Dasytricha increasing and Entodinium decreasing. Using culture independent techniques, Mosoni et al. (2007) examined the effect of YC supplementation on 3 known species of cellulolytic bacteria, *F. succinogenes*, *R. flavefaciens*, and *R. albus,* and found that YC caused 2 to 4-fold increases in *R. flavefaciens* and *R. albus* but had no effect on *F. succinogenes* populations. More recently, Pinloche et al. (2013) examined the effect of YC on the rumen microbiome in early lactation Holstein cows fed to induce SARA. The supplementation of YC produced a 2-fold increase in *Megasphaera spp.* and *Selenomonas spp*., both lactate utilizing bacterial populations, and a 2-fold increase in *Fibrobacter spp*. and *Ruminococcus spp.*, both cellulolytic bacterial populations. Conversely, there was a 25% decrease in *Prevotella spp.* and 7-fold decrease in *Mitsuokella spp.*, both starch-degrading bacterial populations. AlZahal et al. (2014) also demonstrated several fold increases in *F. succinogenes*, *Anaerovibrio lipolytica*, *R. albus*, and anaerobic fungi when active dry *Saccharomyces cerevisiae* was supplemented to lactating Holstein cows fed to induce SARA. All of these results support the mode of action of yeast on rumen function, and in turn, the performance responses of supplemented animals.

Summary

The advent of molecular biological techniques that allow for whole genome analysis of the ruminal microbiome have allowed researchers to examine the effects of dietary changes or additives on whole populations of microorganisms and individual species in relation to the performance responses observed in the host animals. These studies illustrate how the cow has tremendous control over both rumen function and the microbial populations within the rumen and that there is a limited common core bacterial population across groups of cows consuming the same diet. The individual cow control and limited core explain some of the animal to animal variation observed in performance with dietary changes. Future research should focus on understanding how to manipulate this common core microbial population in order to generate consistent responses across a wide group of animals.

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