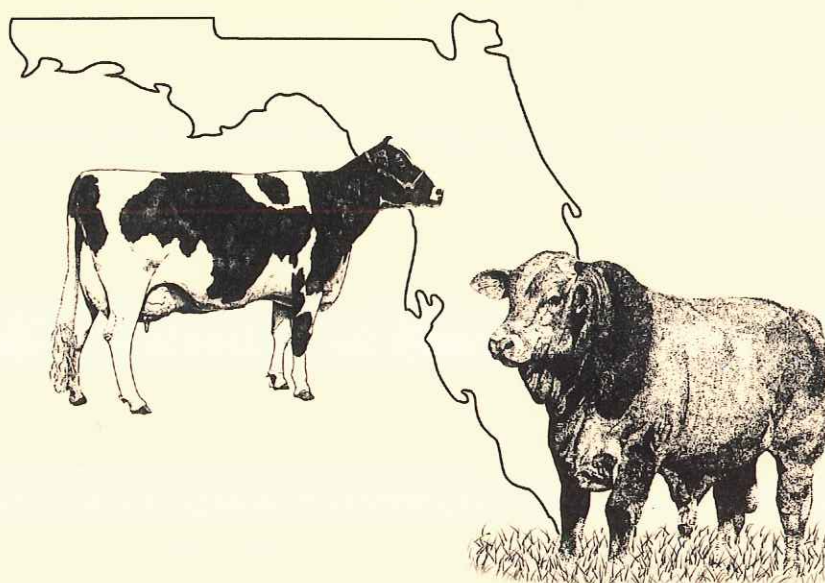


# **2019 Florida Ruminant Nutrition Symposium**

## **30<sup>th</sup> Annual Meeting**



**February 4 - 6, 2019**  
**Best Western Gateway Grand**  
**Gainesville, Florida**

# **PROCEEDINGS**

**UF** | UNIVERSITY of  
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**2019**

**30<sup>th</sup> ANNUAL FLORIDA RUMINANT  
NUTRITION SYMPOSIUM**

**February 4 - 6, 2019  
Best Western Gateway Grand Hotel  
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## Florida Ruminant Nutrition Symposium – February 4 to 6, 2019

**Monday, February 4, 2019** - Mini-Symposium sponsored by Balchem Corporation *"New insights into improved calf performance"*

- 2:00 PM **Dr. Glen Aines**, Balchem Corporation. *Welcome and introductions*
- 2:05 PM **Dr. Bob James**, Down Home Heifer Solutions. *"What are the industry standards for calf performance?"*
- 2:45 PM **Dr. Mike Van Amburgh**, Cornell University. *"Successfully developing a high performing heifer calf"*
- 3:30 PM Refreshment Break
- 3:45 PM **Dr. Charles Staples**, University of Florida. *"Does prenatal choline supplementation play a role in calf performance?"*
- 4:30 PM **Bob Hostetler**, ST Genetics. *"Achieving high calf performance on the farm"*
- 5:15 PM **Dr. Glen Aines**. *Summary and wrap-up*
- 5:30 PM Poolside barbeque

**Tuesday, February 5, 2019** - Pre-Conference Sponsored by DSM Nutritional Products *"Lifetime Performance and Redox Balance Issues in Ruminants"*

- 8:15 AM **Dr. Mark Engstrom**, DSM Nutritional Products. *Welcome and introductions*
- 8:30 AM **Dr. Corwin Nelson**, University of Florida. *"Vitamin supplementation for brood cows in Florida"*
- 9:20 AM **Michael Poindexter**, University of Florida. *"Effect of dietary 25-OH-D<sub>3</sub> on performance and activation of the immune system in dairy cattle"*
- 10:10 AM Refreshment Break
- 10:40 AM **Dr. Lorraine Sordillo**, Michigan State University. *"Redox measures in dairy cows: implications to postpartum health"*
- 11:55 AM Buffet Lunch

**Tuesday, February 5, 2019**

- 1:00 PM **Dr. José E. P. Santos**, University of Florida. *Welcome*
- 1:05 PM **Dr. Nick Place**, University of Florida. *IFAS update*
- 1:10 PM **Dr. Hugh Chester-Jones**, University of Minnesota. *"Calf nutrition and first lactation performance"*
- 1:50 PM **Dr. Gregory Penner**, University of Saskatchewan. *"Short chain fatty acids absorption and metabolism"*

- 2:30 PM **Dr. Rick Grant**, William H. Miner Agricultural Research Institute. *"Feed management: dietary characteristics, feeding environment, and dairy cow feeding behavior"*
- 3:10 PM Refreshment Break
- 3:40 PM **Dr. João Vendramini**, University of Florida. *"Monensin effects on beef cattle grazing warm-season perennial grasses"*
- 4:20 PM **Dr. Reinaldo Cooke**, Texas A&M University. *"Nutritional and management considerations to minimize stress and optimize production efficiency in calf systems"*
- 5:00 PM Welcome reception

### **Wednesday, February 6, 2019**

- 6:30 AM Continental Breakfast
- 8:00 AM **Dr. David Barbano**, Cornell University. *"The use of milk fatty acids as an indication of energy balance in dairy cows"*
- 8:40 AM **Dr. G. Andres Contreras**, Michigan State University. *"Lipid mobilization and inflammation during the transition period"*
- 9:20 AM **Dr. Eduardo Ribeiro**, University of Guelph. *"New insights on the role of essential fatty acids on reproduction in dairy cattle"*
- 10:00 AM Refreshment Break
- 10:30 AM **Dr. Adegbola Adesogan**, University of Florida. *"Technologies for improving fiber utilization by ruminants"*
- 11:10 AM **Dr. T. G. Nagaraja**, Kansas State University. *"A microbiologist's view on improving nutrient utilization in ruminants"*
- 11:50 AM Ruminant Nutrition Symposium Adjourns



## **2019 Symposium Speakers**

### **Guests**

Dr. David Barbano, Cornell University  
Dr. Hugh Chester-Jones, University of Minnesota  
Dr. G. Andres Contreras, Michigan State University  
Dr. Reinaldo Cooke, Texas A&M University  
Dr. Rick Grant, William H. Miner Agricultural Research Institute  
Mr. Bob Hostetler, ST Genetics  
Dr. Bob James, Down Home Heifer Solutions  
Dr. T. G. Nagaraja, Kansas State University  
Dr. Gregory Penner, University of Saskatchewan  
Dr. Eduardo Ribeiro, University of Guelph  
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Dr. Corwin Nelson  
Mr. Michael Poindexter  
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Dr. Charles R. Staples

#### **Department of Agronomy and Range Cattle Research and Education Center**

Dr. João Vendramini

## 30<sup>th</sup> Annual Florida Ruminant Nutrition Symposium

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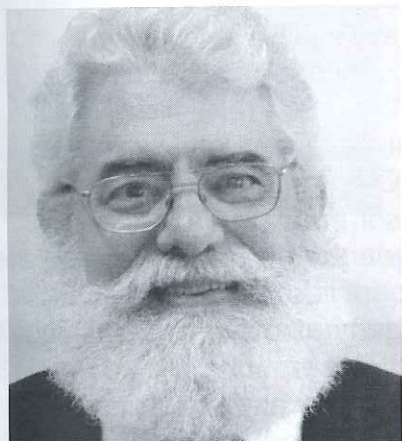


## BIOGRAPHIES



**Dr. Adgebola Adesogan** received his BSc degree in 1988 in Agriculture at the University of Ibadan, Nigeria, and his MSc and PhD degrees in Animal Nutrition at the University of Reading, United Kingdom. He was an Assistant Professor of Animal Nutrition at the University of Wales, UK from 1995 to 2001, and is currently a Professor of Animal Nutrition and Director and Principal Investigator of the Feed the Future Innovation Lab for Livestock Systems. He has served on the Editorial boards of the Journal of Animal Science and other journals and chaired or co-chaired the committees of over 13 PhD students and 6 MS students and mentored over 50 visiting scientists and interns from over 20 countries. He has

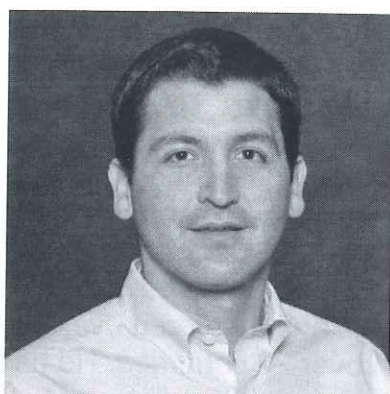
authored or coauthored over 200 scientific publications, received over \$65 million in research grants, and given over 100 international or national seminars in over 25 countries. He has received various awards including the 2006 Graduate Student Mentor of the Year award at University of Florida (UF), the 2007 American Dairy Science Association Pioneer Hi-Bred International Inc. Forage Award, the 2009 UF Research Foundation Professorship award, the 2010 LEAD 21 Award Fellowship from the Florida Agricultural Experiment Station, the Faculty Commons Nilson Award. For outstanding leadership, service and Christian ministry by university faculty and administrators and the 2018 International Educator of the Year award, College of Agriculture, UF.



**Dr. David M. Barbano** is a Professor of Food Sciences at Cornell University and the Director of Northeast Dairy Foods Research Center. David earned his BSc in Biology/Food Science and MSc/PhD in Food Science at Cornell. Dave conducts an applied and basic research program on dairy product manufacturing and milk analysis for dairy herd management. Recently, Dave has focused on developing new measures of cow metabolic health, nutrient utilization, and metabolic stress for dairy herd management using mid-infrared milk analysis. He has been very active in the analytical groups of International Dairy Federation and the Association of Official Analytical

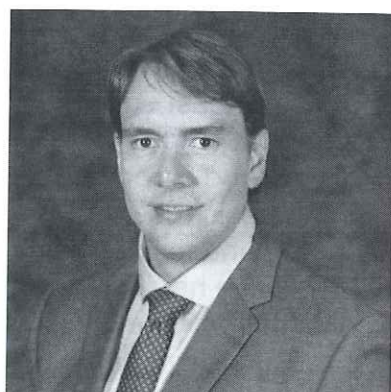
Chemists International for the past 30 years. He has been a member of the American Dairy Science Association since 1974 and is a past president. Honors include: American Dairy Science Association West Agro Award (2008); for Milk Quality Research, Harvey Wiley Award - Association of Official Analytical Chemists International (2010); Award of Excellence - International Dairy Federation (2014); American Dairy Science Association Elanco Award for Excellence in Dairy Science (2015), American Dairy Science Association De Laval Extension Award (2017); American Dairy Science Association - 100 peer reviewed JDS Publications Award (2017); National Cheese Institute - Cheese Laureate Award (2018); Babcock Educator Award - Wisconsin Cheese Makers Association (2018); Award of Merit - American Dairy Products Institute (2018). He feels the best part of his job is working together with students to create new knowledge, technology, and science-based solutions to problems in the dairy industry.





**Dr. Andres Contreras** received his DVM from Universidad Nacional de Colombia. After 3 years of private practice in central Colombia working with tropical cow-calf operations and grazing dairy herds, he served as an intern in the Dairy Internship Program at Michigan State University in East Lansing, MI. Dr. Contreras continued his education at MSU receiving a master's degree in mastitis and milk quality and a PhD in Comparative Medicine and Integrative Biology. His postdoctoral training was at The Center for Integrative Metabolic and Endocrine Research (CIMER) at Wayne State University and focused on the lipolysis-induced

adipose tissue remodeling process and on elucidating the effects of sympathetic innervation on the differentiation of adipocytes in different fat depots. Dr. Contreras' research program is focused on the adaptations of adipose tissue to disease and negative energy balance and its implications to dairy cattle health especially during the periparturient period. Currently his lab is working on the effects of the interactions between adipose tissue immune cells and adipocytes on lipolytic and lipogenic responses around parturition.



**Dr. Reinaldo F. Cooke** is an Associate Professor in the Department of Animal Science at Texas A&M University. Cooke received a BSc (2003) in Animal Sciences from Universidade Paulista, and a MSc (2006) and a PhD (2009) in Animal Sciences from the University of Florida. Prior to joining Texas A&M, Dr. Cooke served Oregon State University as Assistant and Associate Professor from 2009 to 2017. Cooke's academic program is geared toward addressing the needs of the Texas, US, and worldwide beef industries. His research efforts focus on management strategies to improve productive efficiency in beef cattle operations, including

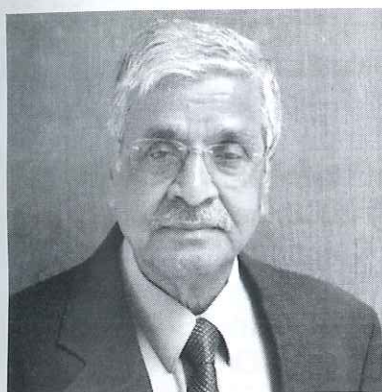
nutrition, health, growth, and reproductive responses in *Bos indicus* and *B. taurus*. To date, Dr. Cooke has authored/co-authored 100 journal articles and 3 book chapters, delivered more than 150 extension presentations in local, national, and international events, and secured \$4 million in extramural research funding. Dr. Cooke has mentored seven PhD students, ten M.Sc., forty research interns, and two post-doctoral students. Dr. Cooke serves in the American Society of Animal Sciences (ASAS) Western Section as President-Elect, Section Editor for the Journal of Animal Science and received the ASAS Early Career Achievement Award in 2018, ASAS Western Section – Extension Award in 2017, and ASAS Western Section - Young Scientist Award in 2016.





**Dr. Rick Grant** was raised on a dairy farm in northern New York State. He received a BSc degree in Animal Science from Cornell University, a PhD from Purdue University in ruminant nutrition, and held a post-doctoral position in forage research at the University of Wisconsin-Madison from 1989 to 1990. From 1990 to 2003, Rick was a professor and extension dairy specialist in the Department of Animal Science at the University of Nebraska in Lincoln. Since February of 2003, he has been President of the William H. Miner Agricultural Research Institute in Chazy, NY, a privately funded educational and research institute

focused on dairy cattle, equine, and crop management. Rick's research interests focus on forages, dairy cattle nutrition, and cow behavior. He has been the recipient of the Pioneer Hi-Bred International Forage Award in 2010 and the Nutrition Professionals Applied Dairy Nutrition Award in 2015.



**Dr. T. G. Nagaraja** is a University Distinguished Professor of Microbiology in the Department of Diagnostic Medicine/Pathobiology in the College of Veterinary Medicine. His research expertise is in gut microbiology of cattle. His research has focused primarily on role of rumen microbes in function and dysfunction of the rumen, and on food borne pathogens, particularly Shiga toxin-producing *Escherichia coli* and *Salmonella* in cattle. His teaching responsibilities include Veterinary Bacteriology and Mycology Lecture and Laboratory for the sophomore DVM students, Ruminant Digestive Physiology for the freshman

DVM students, two graduate courses on the rumen, Rumen Metabolism and Rumen Microbiology. Additional responsibilities include serving as the Director of the PhD Program in Pathobiology and the MS Program in Veterinary Biomedical Science in the College of Veterinary Medicine. Nagaraja's research is a blend of basic and applied studies and involves collaborative interactions with epidemiologists, molecular biologists, pathologists, and ruminant nutritionists. Nagaraja and his associates have made significant contributions in the following areas: Use of ionophore antibiotics in cattle; Causes, pathogenesis and vaccine development for liver abscesses in feedlot cattle; causes and preventions of ruminal disorders, such as acidosis and bloat; Ecology of Shiga toxin-producing *E. coli* and *Salmonella* in cattle; and, Antimicrobial resistance and Antimicrobial alternatives. His extramural research support (over \$10 million) has been predominantly from the USDA and Animal Health companies. He has mentored 19 PhD, 18 MS, and 3 MPH students and several post docs and visiting scientists. His research has resulted in seven US patents. Nagaraja and his associates have published 18 book chapters, 14 review papers, 5 symposia proceedings, and 212 peer-reviewed journal papers.





**Dr. Greg Penner** is an Associate Professor in the Department of Animal and Poultry Science at the University of Saskatchewan. He obtained his BSA and MSc degrees from the University of Saskatchewan in 2004, and his PhD from the University of Alberta in 2009. His research primarily focuses on understanding the regulation of absorptive barrier function of the gastrointestinal tract in ruminants. Notable accomplishments include the development of indwelling pH measurement systems that have been adopted by the research community worldwide. Dr. Penner has a well-funded research program, has published 74

reviewed papers, provided numerous invited presentations and, in 2012, he received the Canadian Society of Animal Science Young Scientist Award. Greg also has an active extension program helping to communicate research results to end users and serves as the co-chair for the Saskatchewan Beef and Forage Symposium.



**Dr. Eduardo Ribeiro** grew up on a family farm in a small town located in southern Brazil. In 2008, he graduated with a degree in Veterinary Medicine at the Santa Catarina State University, and, in 2009, he moved to the United States to start his graduate studies in the Department of Animal Sciences at the University of Florida under supervision of Dr. José E. P. Santos. He completed his MSc degree in 2011 and his PhD degree in 2015. In 2016, Dr. Ribeiro joined the Department of Animal Biosciences at the University of Guelph as an Assistant Professor in Reproductive Physiology. His research seeks to understand the nature and causes of

pregnancy losses in bovine, and to develop strategies that ultimately improve pregnancy survival and reproductive efficiency in dairy herds.

She also has served on many national committees and has received several awards for her research on bovine immunology and mastitis control.



**Dr. João Vendramini** received his BSc degree in agronomy from the University of São Paulo, the MSc degree in Animal Sciences from the same institution, and the PhD in forage management at the University of Florida in 2005. He was an Assistant Professor and Forage Specialist at Texas A&M University from 2005 to 2006 before taking his current research and extension appointments at the University of Florida Range Cattle Research and Education Center, Ona, FL. Dr Vendramini's program is dedicated to forage management with emphasis on sub-tropical production systems. The major area of interest is forage-livestock

interface and the impact of forage management on forage and animal production. Dr. Vendramini's research program has generated 5 book chapter; 111 refereed journal articles, 52 extension articles, and 150+ abstracts in professional meetings. He has been the principal investigator or co-principal investigator on grants totaling \$1,200,000 and currently he is the chair or co-chair of 2 graduate students and serves as a member of an additional 6 committees. He received the 2010 Merit Award – American Forage and Grassland Council, 2011 Florida Cattlemen's Association Researcher of the Year, 2011 Florida Association of County Agriculture Agents Outstanding Specialist, and the 2017-2020 UF/IFAS Term Professorship Award. Dr. Vendramini is a member of the American Society of Animal Sciences, American Registry of Animal Science Professionals, American Society of Agronomy, Crop Science Society of America, American Forage and Grassland Council, and Florida Cattlemen's Association.



# Short Chain Fatty Acid Absorption and Metabolism

Gregory B. Penner<sup>1</sup>

*Department of Animal and Poultry Science, University of Saskatchewan*

## Introduction

Ruminants have a unique ability to derive energy from complex carbohydrates as microbes ferment the carbohydrates yielding short-chain fatty acids (**SCFA**). Short-chain fatty acids have been estimated to provide up to 75% of the total metabolizable energy (Bergman, 1990) for cattle. Thus, it is not surprising that diets that are readily fermentable promote greater production of SCFA also drive productivity outcomes (e.g. milk production) to a greater extent than less fermentable diets (Kolver and de Veth, 2002; Oba and Allen, 2003a; 2003b). However, as weak acids, SCFA will dissociate in the rumen releasing a proton thereby decreasing ruminal pH under most circumstances. This highlights the double-edged sword where promoting SCFA production leads to greater energy supply, but too much SCFA or a rate of SCFA production that exceeds the ability to neutralize the protons (acid) reduces ruminal pH and can lead to ruminal acidosis. Understanding SCFA absorption in relation to ruminal pH and factors that can stimulate SCFA absorption to increase energy supply are key to enhancing production responses.

## Mechanisms of SCFA Absorption and the Linkage to Ruminal pH

The ruminal contents are highly stratified due to the compartmentalization of the reticulo-rumen and the nature of the digesta within. Short-chain fatty acid production occurs primarily at the rumen-fluid rumen-mat interface and studies have consistently identified the rumen-fluid rumen mat interface as the region that has the lowest pH (Lui et al., 2009; Storm and Kristensen, 2010) and greatest SCFA concentrations (Storm and Kristensen, 2010). While this is logical, it presents a challenge in terms of SCFA absorption: for SCFA to be absorbed, they must be exposed to the ruminal epithelium. Thus, the stratification of the rumen provides a diffusional gradient that must be overcome (Storm and Kristensen, 2010). The primary mechanism to ensure SCFA are exposed to the ruminal epithelium is through rumen motility. However, few studies have considered rumen motility as a factor promoting SCFA absorption (Storm and Kristensen, 2010).

## Short Chain Fatty Acids Absorption

Once SCFA are exposed to the ruminal epithelium, they are capable of being absorbed. The ruminal epithelium is a complex tissue consisting of 4 cell strata with many cell layers. The structural complexity creates a physical barrier from the ruminal environment and metabolically active layers that can absorb and metabolize SCFA. To better understand SCFA absorption, it is important to consider the histological arrangement of the ruminal epithelium. The outer-most layer of the ruminal epithelium is

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the stratum corneum. The cells within the stratum corneum are highly keratinized and not metabolically active. These protect the underlying strata from physical abrasion and likely contribute very little as a barrier or promoting factor for SCFA absorption. The next layer is the stratum granulosum. This stratum is characterized by cells that are increasingly keratinized and have few intracellular organelles. However, the stratum granulosum is the primary site for tight-cell junctions and thus acts as a physical barrier to ensure absorption of SCFA occurs while preventing passage of non-desired molecules. The next layer is the stratum spinosum. The cells within the stratum spinosum are metabolically active and have gap junctions that serve to facilitate cell-to-cell communication and exchange of ions. Finally, the most inner layer is the stratum basale. The stratum basale are highly active cells and are the layer where cell division occurs. The cell division is necessary to provide new cells that mature and differentiate and migrate toward the stratum corneum.

Given the structural complexity of the ruminal epithelium, 2 barriers on the ruminal epithelium can be highlighted. Firstly, SCFA must cross the apical side of the ruminal epithelium (stratum granulosum) to be absorbed within the ruminal epithelium and secondly, SCFA and their metabolites must be exported out of the ruminal epithelium into portal blood so that they can contribute to the systemic energy supply of the host. Fortunately, the ruminal epithelium is highly vascularized promoting blood flow and the movement of SCFA and metabolites from the epithelium into portal blood flow.

Although mechanisms of SCFA have been investigated since the 1940's (Dijkstra et al., 1945; Masson and Phillipson, 1951; Ash and Dobson, 1963), it was largely assumed that SCFA absorption occurred via passive diffusion. The suggestion for passive diffusion was based on the inability to achieve saturation of SCFA absorption with increasing SCFA concentration and that reducing pH increased SCFA absorption (Dijkstra et al., 1993; López et al., 2003; Graham et al., 2007). It is important to recognize that as pH decreases, the proportion of SCFA in the undissociated state increases and that only undissociated SCFA are permeable to cross the lipid bilayer of cells (Walter and Gutknecht, 1986; Dijkstra et al., 1993; López et al., 2003). Thus, a reduction in pH would increase the proportion of undissociated SCFA that could then freely diffuse across the rumen epithelium.

There are numerous theoretical constraints for a model solely relying on passive diffusion. Firstly, the proportion of SCFA in the undissociated state ( $pK_a = 4.8$ ) is low under normal pH conditions in the rumen. Even with pH values of 5.8, more than 90% of the SCFA would be in the dissociated state. Previous researchers had suggested that there was an acidic pH microclimate on the luminal side of the ruminal epithelium (Graham and Simmons, 2005) allowing for a greater proportion of SCFA in the undissociated state immediately adjacent to the epithelium. However, basic apical pH values have been reported (7.47 to 7.68) depending on the incubation conditions (Leonhard-Marek et al., 2006). Lipophilicity constants also suggest that butyric acid should be absorbed about 10 times more rapidly than acetic acid (Walter and Gutknecht, 1986). However, similar fractional absorption rates have been reported among SCFA in vitro (Aschenbach et al., 2009) and when differences are found (Dijkstra et al., 1993; López et al., 2003), they are not consistent with the increase predicted based on lipophilicity. Moreover, a recent



showed that although the concentration of SCFA increased from 10 to 50 mM, the rates of acetate and butyrate absorption only increased by 2.1 and 2.4 times for acetate and butyrate, respectively (Schurmann et al., 2014). The model of passive diffusion also does not consider how SCFA are transported across the basolateral (blood facing) side of the epithelium.

A simplified model showing the current understanding of the mechanisms involved in SCFA absorption and how the absorption of SCFA contributes to the stabilization of ruminal pH is depicted in Figure 1 (Aschenbach et al., 2011). The predominant mechanisms include: 1) SCFA<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> anion exchange; 2) passive diffusion; 3) nitrate-sensitive SCFA absorption; 4) proton-coupled SCFA<sup>-</sup> transport; and 5) electrogenic SCFA transport. While these are the major absorption mechanisms, other processes such as Na<sup>+</sup>/H<sup>+</sup> exchange, and bicarbonate import into the cells are required to enable the maintenance of intracellular pH and to promote SCFA absorption. These pathways have been reviewed extensively in Aschenbach et al. (2011).

Much of the SCFA absorption occurs through anion exchange where SCFA<sup>-</sup> are absorbed in exchange for release of HCO<sub>3</sub><sup>-</sup> into the rumen and further the SCFA<sup>-</sup> can cross the basolateral membrane in exchange for HCO<sub>3</sub><sup>-</sup> import into the cell (Bilk et al., 2005; Aschenbach et al., 2009; Penner et al., 2009b). Based on the available data, approximately 42 to 57%, 0 to 14%, and 29 to 59% of the acetate transport relies on bicarbonate-dependent, nitrate-sensitive, and passive diffusion, respectively (Penner et al., 2009a; Schurmann, 2013). For butyrate, the proportion accounted for by bicarbonate-dependent transport, nitrate-sensitive transport, and passive diffusion are 24 to 46, 0 to 4, and 25 to 76%, respectively (Penner et al., 2009a; Schurmann, 2013).

In the rumen, the majority of the SCFA will be in the dissociated state (SCFA<sup>-</sup>). Absorption of SCFA<sup>-</sup> occurs in exchange for HCO<sub>3</sub><sup>-</sup> in an electro-neutral process that is mediated by a number of anion exchangers (Bilk et al., 2005; Aschenbach et al., 2009; Penner et al., 2009b). This mechanism provides a source of bicarbonate to the ruminal environment where it can neutralize a proton via the carbonic anhydrase reaction producing carbon dioxide and water. Driving forces for bicarbonate-dependent transport include the concentration of ruminal SCFA and ruminal pH. In fact, the bicarbonate-dependent SCFA absorption increases with increasing luminal SCFA concentration and with decreasing ruminal pH (Aschenbach et al., 2009). The bicarbonate facilitating this transport does not seem to occur in the cytosol, but rather is transported from arterial circulation into the cell (Sehested et al., 1999; Aschenbach et al., 2009). There are several bicarbonate transporters including anion exchangers on the basolateral (blood-facing) side that may also help to export SCFA<sup>-</sup> out of the cell and into arterial blood. Thus, it appears that this transport process is crucial in terms of helping to regulate ruminal pH (Penner et al., 2009a) and exporting SCFA to be metabolized by other tissues.

When H-SCFA are absorbed via passive diffusion, 1 proton is removed from the ruminal digesta; however, H-SCFA will dissociate in the cytosol releasing SCFA<sup>-</sup> and H<sup>+</sup>. The proton (H<sup>+</sup>) released must be removed from the cell or neutralized in order to maintain intracellular pH and tissue integrity. Transporters involved in the regulation of intracellular



pH include the sodium/hydrogen exchangers (**NHE**) that export protons back to the or into extra-cellular spaces. In addition to NHE, the monocarboxylate transporter has been shown to be localized on the basolateral membrane (blood facing; Graham Simmons, 2007) and can facilitate the removal of a proton along with metabolic products of SCFA metabolism such as ketone bodies and lactate (Müller et al., Kirat et al., 2006). Thus, the direction of proton export has major implications for whether passive diffusion contributes to the stabilization of ruminal pH. For example, if the is exported back into the rumen contents as a strategy to maintain intracellular pH, would be no net proton removal from the rumen and therefore ruminal pH would not be affected. Interestingly, the expression and activity of NHE in ruminal epithelia increase when highly fermentable diets are fed (Etschmann et al., 2009; Yang et al., Schurmann, 2013). However, due to the complexity of the transport mechanisms involved and the regulation of their activity, it is very difficult to quantify or predict the proportion of protons recycled back to the lumen relative to those that account for permanent removal from the ruminal contents. That said, it is clear that under some circumstances passive diffusion does contribute to the removal of protons from the rumen (Penner et al., 2009).

In addition, it is now known that there is a nitrate-sensitive transport pathway for SCFA. This process occurs both in the presence and absence of bicarbonate (Aschenbach et al., 2009), but currently the transporters involved are not known. From unpublished work (K. Wood, J.R. Aschenbach, F. Stumpff, and G.B. Penner) has shown a clear inhibitory effect with increasing concentrations of nitrate for acetate but not for butyrate. Future studies are required to improve our understanding of this transport mechanism and its regulation. Finally, electrogenic SCFA<sup>-</sup> transport has been documented (Stumpff et al., 2009; Georgi et al., 2013). This transport process is thought to be mediated by maxi-anion channels but the total contribution to SCFA transport is not currently known.

### **Evidence Linking SCFA Absorption to the Stabilization of Ruminal pH**

Early studies (Masson and Phillipson, 1951; Dobson and Ash, 1963; Gäbel et al., 1991) had suggested that SCFA absorption could be one mechanism for the stabilization of rumen pH. However, the first evidence supporting the pH stabilizing effect of SCFA absorption was provided by Resende Júnior et al. (2006). In that study moderate to high (0.43) positive correlations between the fractional rate of SCFA clearance and ruminal pH were observed suggesting that greater rates of SCFA clearance corresponded to improved ruminal pH. Resende Júnior et al. (2006) further evaluated whether the effect on pH was due to absorption of SCFA across the rumen wall or the passage of SCFA out of the rumen finding that both mechanisms were positively related to ruminal pH. In another study Penner et al. (2009b), reported negative associations between ruminal pH and expression of a number of genes involved in SCFA metabolism and the severity of ruminal acidosis for dairy cows fed a diet containing 64% concentrate. While these studies suggest relationships between ruminal pH or the severity of ruminal acidosis and the absorption of SCFA or indicators for intra-epithelial metabolism of SCFA, they cannot prove



SCFA absorption improves ruminal pH nor can they elucidate how the pathway of SCFA and type of SCFA affect ruminal pH.

Penner et al. (2009a) conducted a study to determine the relationship between the uptake of SCFA and the severity of ruminal acidosis. In that study, ruminal acidosis was induced in 17 lambs using an oral glucose drench (5 g glucose/kg BW). Based on the ruminal pH response over 3 hours after the drench, lambs were assigned to 1 of 2 classifications; non-responders (**NR**; the 7 lambs that had the least ruminal pH reduction) or responders (**RES**; the 7 lambs that had the greatest reduction in ruminal pH following the challenge). To evaluate the relationship between ruminal pH and SCFA absorption, the rumen epithelium was collected and the uptake of acetate and butyrate was measured *ex vivo*. Results from the NR and RES lambs were compared to a group that was not exposed to an acidotic challenge (**SHAM**). Ruminal pH differed between sheep classified as NR (67.8 min), RES (153 min) and SHAM (1.1 min) as did the uptake of acetate and butyrate. It is important to note that we assumed that the acidotic challenge imposed did not compromise the ruminal epithelium as acetate and butyrate uptake did not differ between the RES and SHAM treatments. Interestingly, we found that epithelia from NR sheep had a greater rate of total acetate and butyrate uptake than RES indicating that the improved ruminal pH response could be attributed to greater capability for SCFA uptake. In addition, retrospective correlation analysis showed that acetate and butyrate uptake was also positively related to the mean pH prior to the acidotic challenge. This is the only study (Penner et al., 2009a) that has provided comprehensive data demonstrating that the rate of acetate and butyrate uptake has a substantial effect on ruminal pH homeostasis.

As mentioned above, the pathway of SCFA absorption may play a role in the stabilization of ruminal pH. In addition to total uptake, Penner et al. (2009a) also reported that the main mechanisms facilitating acetate and butyrate uptake were different between NR and RES. For acetate, the bicarbonate-dependent and bicarbonate-independent nitrate-sensitive transport was greater for NR than RES. As mentioned above, with the bicarbonate-dependent transport, bicarbonate secretion and acetate absorption are coupled. Interestingly, for butyrate, bicarbonate-independent (passive diffusion) uptake was higher for NR than RES. Collectively these data indicate that the pathway of SCFA absorption may differ based upon the type of SCFA and thus the relative contribution towards the stabilization of ruminal pH may also differ. For example, acetate is not as lipophilic as butyrate and thus protein-mediated pathways contribute substantially towards its uptake. This is important as the bicarbonate-dependent pathway would also provide bicarbonate to buffer the rumen contents (Aschenbach et al., 2009). In contrast, butyrate has a greater potential for diffusional uptake (Walter and Gutknecht, 1986). Thus, factors promoting a concentration gradient between the rumen, cytosol, and blood should promote absorption (Gäbel et al., 2002). The suggestion that intracellular metabolism enhances butyrate absorption is in alignment with Gäbel et al. (2001) and previously reported negative correlations between the expression of genes involved in butyrate metabolism and the severity of ruminal acidosis (Penner et al., 2009b). Furthermore, we found that NR sheep had greater serum  $\beta$ -hydroxybutyric acid (**BHBA**; a metabolite of butyrate metabolism) than RES sheep after the 180 min acidotic challenge (Penner et al.,



2009a). The increase in serum BHBA may also indicate that for butyrate, metabolism to ketone bodies and export from the cell via MCT may help to regulate ruminal pH.

### Nutritional Modulation of SCFA Transport

Given the importance of SCFA transport towards meeting the energy requirement and stabilization of ruminal pH, several studies have investigated whether dietary or feeding management can modulate the response. Interestingly, past studies have clearly demonstrated that SCFA can be manipulated through management and dietary interventions.

#### ***Low Feed Intake and Feed Deprivation Decrease SCFA Absorption***

The vast majority of current research has focused on rumen epithelial adaptation from an anabolic perspective, however, in times of scarcity or in response to a nutritional insult, the adaptive response certainly includes regression. In fact, the long-term changes induced by a low plane of nutrition have been shown to decrease gut mass and reduce O<sub>2</sub> consumption by visceral tissue, and reduce SCFA absorption (Doreau et al., 1997). Understanding how the ruminal epithelium responds to reductions in SCFA exposure due to a transient low feed intake and, more importantly, the timeline required for the epithelium to return to the pre-restriction function is needed to develop feeding strategies and mitigate disorders associated with digestive upset.

Albeit unintentional and generally short in duration, beef and dairy cattle are exposed to periods of feed restriction or complete feed deprivation. Examples include during weaning, transportation, prior to and immediately after parturition, immediately following digestive upset, while experiencing heat stress, and in association with metabolic disorders and infection. Gäbel et al. (1993) demonstrated that 48-h of complete feed reduced SCFA, Na<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> absorption by approximately 40 to 60%. It is important to note that these changes were likely due to a reduction in the functional capacity and blood flow rather than changes induced by epithelial surface area. More recently, the effect of the severity of short-term feed restriction, rather than complete feed deprivation, has been investigated (Zhang et al., 2013a). In this study, 18 heifers were fed *ad libitum* and then allocated feed equating to 75, 50, or 25% of their *ad libitum* dry matter intake (DMI) for a period of 5 d. A 5-d feed restriction period, regardless of the severity, tended ( $P = 0.09$ ) to decrease total SCFA absorption and decreased acetate absorption. Additionally, heifers restricted to 50 and 25% of *ad libitum* intake tended ( $P = 0.07$ ) to have lower rates for total SCFA and acetate absorption compared to those restricted to 75% of *ad libitum* intake. It does not appear that shifting the dietary forage-to-concentrate ratio will mitigate this effect despite expected changes in fermentability and ruminal retention time (Albornoz et al., 2013a). For example, when cattle were restricted to 25% of their *ad libitum* intake for 5 d, the total SCFA absorption rate decreased by 120 mmol/h relative to baseline measurements and did not differ between heifers fed a diet consisting of 92% forage vs. those fed 60% forage (Albornoz et al., 2013a). Thus, it appears that reductions in ruminal epithelial function occur rapidly in response to lower energy intake.



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A rapid reduction in ruminal epithelial function may be a compensatory mechanism to reduce energy expenditure by ruminal tissue (Zhang et al., 2013a) during periods of low energy intake. However, given the transient nature of low feed intake under conventional feeding systems, a rapid increase in epithelial function corresponding to increased energy intake would be desirable. Zhang et al. (2013b) provided heifers *ad libitum* access to feed, without changes in the diet composition, after a 5-d period of feed restriction. That study reported two important findings: 1) return to *ad libitum* feeding without dietary change induced ruminal acidosis, and 2) that time to recover absorptive function increased with increasing severity of feed restriction. In fact, heifers restricted to 25% of their *ad libitum* intake required 3 weeks for SCFA absorption rates to recover, whereas those restricted to 75% of their *ad libitum* intake recovered within 1 wk. The delayed recovery response suggests that at least a portion of the response is mediated by the epithelia and not solely due to changes in blood flow. Interestingly, the recovery response appears to be hastened when cattle are fed greater proportions of concentrate prior to dietary restriction and greater proportions of forage after feed restriction (Albornoz et al., 2013b).

#### Ruminal Acidosis Compromises SCFA Absorption

Providing adequate time for dietary adaptation has been recommended as a strategy to reduce the risk for ruminal acidosis. It is evident that repeated exposure to sub-acute ruminal acidosis or a single exposure to acute ruminal acidosis may also negatively affect SCFA absorption. Dohme et al. (2008) reported that the response to repeated ruminal acidosis inductions increased in severity with each consecutive challenge despite the cows consuming less grain during consecutive challenges. While there may be a number of reasons behind this response, a decrease in SCFA absorption is highly plausible because previous studies have shown that at similar pH values ( $< 5.4$ ) epithelial damage was induced (Steele et al., 2009) and ion transport was impaired (Gaebel et al., 1987; Gaebel et al., 1988; Gaebel et al., 1989). That said, it is not clear whether adaptation reduces the risk for ruminal acidosis. In a recent study, we compared whether cattle fed a high-grain diet (81% barley grain, 10% vitamin and mineral supplement, 9% barley silage) for 34 d were more resistant to ruminal acidosis than cattle fed the same diet but for only 8 d (Schwaiger et al., 2013a,b). Ruminal acidosis was induced by restricting feed intake on the d before the challenge and the challenge itself included an intraruminal infusion of ground barley grain. There were no differences observed for the risk or severity of ruminal acidosis between short-adapted and long-adapted cattle. However, we did observe that ruminal pH recovered more rapidly in long-adapted cattle than short-adapted cattle. Interestingly, long-adapted cattle also had greater lactate absorption than short-adapted cattle immediately following the challenge.

While the total SCFA absorption rate was not different between the short- and long-adapted cattle, it was very clear that induction of ruminal acidosis decreased SCFA absorption (Schwaiger et al., 2013a,b) when measured 2 d following induction of ruminal acidosis but not when measured 9 d after the induction of ruminal acidosis. Moreover, there appears to be a compensatory shift in ruminal buffering strategies such that



absorption is reduced following a bout of ruminal acidosis while at the same time, saliva production increases. Thus, it appears that ruminal acidosis impairs SCFA absorption but the recovery following a bout of ruminal acidosis may be rapid and that cattle may increase salivary buffer supply to compensate for the reduction in SCFA absorption. The negative effect of severely low ruminal pH on SCFA absorption is supported by previous work in vivo (Krehbiel et al., 1995) and in vitro (Wilson et al., 2012).

### **Promoting SCFA Absorption**

To apply the concept of nutritional challenges within the feedlot sector, a study was conducted to evaluate strategies to accelerate recovery of gastrointestinal tract following a nutritional challenge (Penner et al., unpublished). In this study, 32 lambs were assigned to 1 of 4 treatments. The treatments consisted of a finishing ration (9% barley silage, 79% barley grain, and 12% of a barley-based mineral and vitamin supplement) throughout the study (**CON**) or lambs that were fed the finishing ration but exposed to a 3-d period of low feed intake (**LFI**) at 50% of voluntary intake and then 1 of 3 recovery treatments. The recovery period was 5-d. To evaluate the recovery response after low feed intake, lambs were either fed the finishing ration (**FIN**), or 1 of 2 diets in which the proportion of barley silage was increased to 20% at the expense of barley grain. This approach is commonly referred to as a 'storm' diet in the feedlot sector (**STORM**). The second 'storm' diet also included a dietary additive of rumen protected betaine (0.7% of DM), superoxide dismutase (0.01% of DM) as an antioxidant, and Na-butyrate (0.2% DM). Betaine has been reported to help support gastrointestinal tract function during coccidia challenges (Kettunen et al., 2001; Fetterer et al., 2003), and superoxide dismutase has been reported to improve gastrointestinal tract function in mice (Vouldoukis et al. 2004). Finally, butyrate has been shown to induce positive effects at low doses (Gorka et al., 2007; Kowalski et al., 2015). We observed that the CON group did not change DMI throughout the study, thereby serving as an appropriate control as they were not exposed to a low feed intake challenge. Interestingly, lambs fed the STORM or STORM plus additive diets during recovery increased DMI relative to that during low feed intake, while lambs fed the FIN diet did not increase DMI during recovery. This suggests, that increasing the proportion of forage after a period of LFI can help recovery of DMI when fed finishing diets. While all treatments, except the CON, had lower ruminal pH during recovery than during the LFI challenge, the STORM and STORM plus additive diets had numerically greater ruminal pH during the 5-d recovery than lambs provided the finishing diet. We also found that lambs fed the STORM plus additive diet tended to have greater rates of acetate absorption and had greater butyrate absorption in the recovery period than the other treatments. This study demonstrated that moderate increases in the forage proportion can help cattle recover after a period of LFI, even with finishing diets, and that provision of additives reported to accelerate gastrointestinal tract function can help the recovery response. Future research is needed to evaluate which additives are most beneficial to improve the recovery of the gastrointestinal tract.

### **Dietary Fatty Acid Supply and Composition Affect SCFA Absorption**

Dietary fatty acids are often used to increase energy density of the diet and can modulate composition of tissues (Owens and Gardner, 2000). However, we are not aware of any studies that have evaluated whether ruminal epithelial composition can be manipulated and whether changes in composition affect SCFA absorption. Twenty-one Holstein steers ( $194 \pm 10.7$  kg) were randomly assigned to the control (**CON**; contained 2.2% ether extract) or one of two lipid treatments (contained 5% ether extract) utilizing saturated (**SAT**) or unsaturated sources (**UNSAT**) of lipid (Verdugo and Penner, unpublished). The SAT lipid sources were primarily from tallow and palmitic acid whereas the UNSAT was provided from flaxseed and Ca salts of fatty acids. After 30 d, calves were killed and samples of ruminal digesta, blood, and ruminal tissue were collected for fatty acid analysis and ruminal tissues were also used for *ex vivo* measurement of acetate, propionate, and butyrate uptake and flux. We observed that inclusion of lipid increased ( $P < 0.01$ ) the concentration of fatty acids in ruminal fluid, but SAT and UNSAT did not differ. Feeding UNSAT decreased the proportion of saturated FA and increased the proportion of mono and polyunsaturated fatty acids in ruminal fluid. The changes in ruminal fluid also were reflected in plasma and ruminal tissue. The ruminal epithelial concentration of fatty acids tended ( $P = 0.10$ ) to be greater for calves fed lipid and for calves fed UNSAT vs. SAT ( $P = 0.06$ ). Interestingly, calves fed supplemental lipid had greater ( $P = 0.03$ ) butyrate uptake than CON, and butyrate uptake was 44% greater for SAT than UNSAT ( $P < 0.01$ ) suggesting that fatty acid supply and type can modulate SCFA absorption.

### **Conclusions**

Short-chain fatty acid absorption clearly helps to stabilize ruminal pH by either removing protons with passive diffusion or by the secretion of bicarbonate with anion exchange mechanisms. Interestingly, the relative contribution of individual pathways of SCFA absorption differ based on the type of SCFA absorbed and the contribution of salivary bicarbonate and epithelial buffering towards stabilization of ruminal pH appear to be affected by ruminal pH itself. A number of factors such as feed restriction and ruminal acidosis negatively affect SCFA absorption. More recent research has also highlighted that nutritional manipulation can enhance SCFA absorption providing a strategy to help support gastrointestinal function and potentially increase productivity.

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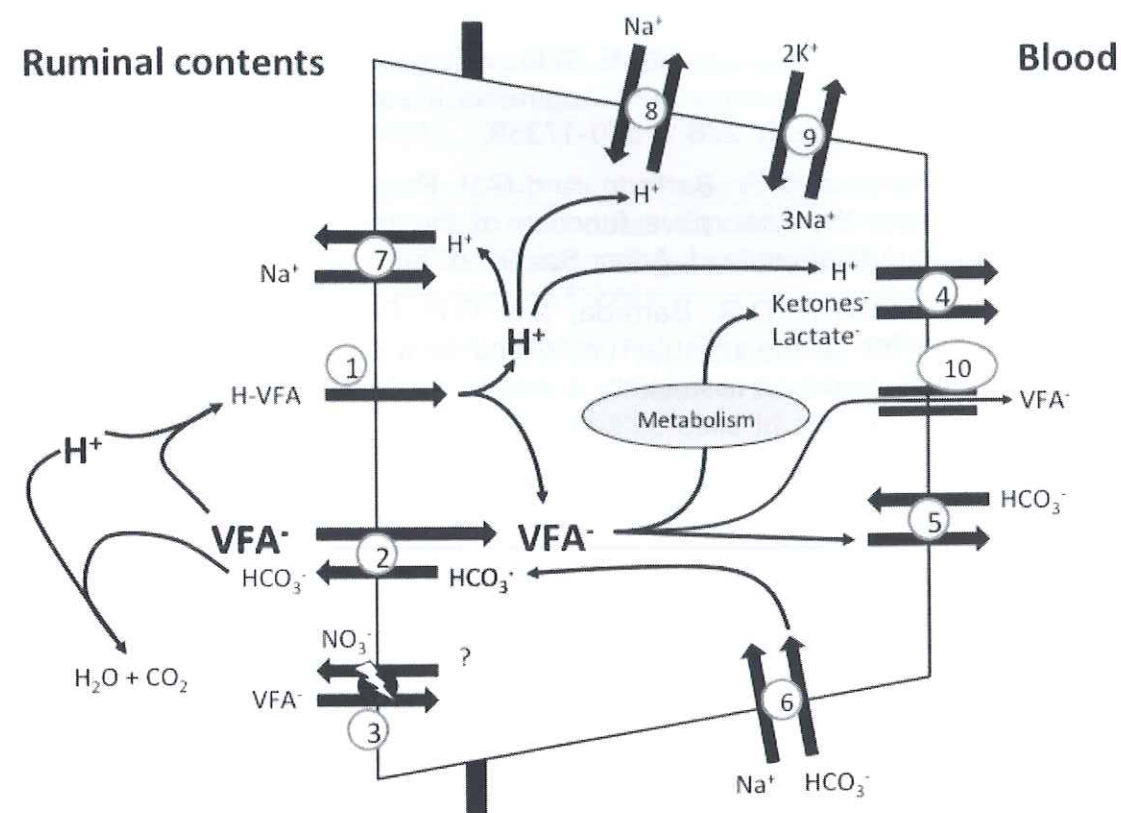


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## SESSION NOTES



**Figure 1.** Partial model depicting the current understanding for SCFA absorption in relation to the stabilization of ruminal pH. 1) Diffusional absorption of SCFA facilitates the removal of a proton associated with the SCFA. This proton will rapidly dissociate in the cytosol where it can be exported by sodium/hydrogen exchanges (7, 8) or coupled with metabolites of SCFA (e.g. ketone bodies and lactate) via the monocarboxylate transporter (4). Dissociated SCFA can be absorbed in an anion exchange mechanism thereby providing a source of bicarbonate to the ruminal contents (2). This bicarbonate can then neutralize a proton through the carbonic anhydrase reaction thereby stabilizing ruminal pH. The bicarbonate supply to the epithelia is derived from blood (5, 6). The SCFA can also be absorbed via a nitrate sensitive pathway (3) and can be exported into blood via a voltage-gated channel (10). Note, the model does not show the structural complexity of the ruminal epithelia including the number of strata and cells within strata. Adapted from Aschenbach et al. (2011).



## Feeding Management: Dietary Characteristics, Feeding Environment, and Dairy Cow Feeding Behavior

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### Introduction

Providing an optimal feeding environment enhances the cow's response to her diet. Ensuring feed availability is particularly critical - herds that routinely feed for refusals and practice consistent feed push-up average about 1.4 to 4.1 kg/d more milk than herds that do not (Bach et al., 2008). Few management factors elicit that magnitude of response, and any assessment of feeding management should begin with feed availability.

When cattle are grouped, competition at the feed bunk is inevitable. Even with unlimited access to feed, cows interact in ways that give some an advantage over others (Olofsson, 1999). Consequently, the management goal is not to eliminate competition at the feed bunk, but rather to control it. Key factors that must be optimized to encourage desirable feeding behavior and optimal intake of a well-formulated ration include:

- Adequate feed availability and accessibility;
- Competition that doesn't hinder access to feed; and
- No restrictions on resting or ruminating activity.

In addition, based on research to-date and practical on-farm observations, recommended feeding management practices include (Grant, 2016): 1) providing consistent feed quality and quantity along the entire length of the feed bunk, 2) keeping bunk stocking density  $\leq 100\%$  ( $\geq 60$  cm/cow), 3) feeding total mixed ration (TMR) 2x/day, 4) ensuring that feed is pushed-up during the 2 hours after feed delivery, 5) targeting approximately 3% feed refusals for cow groups except for fresh pen, which should be closer to 7%, and 6) making certain that the feed bunk is empty no more than 3 h/d (ideally never).

This paper focuses on 1) recent research conducted at Miner Institute on the influence of stocking density and its interactions with key components of the diet and feeding environment, and 2) re-assessing industry norms for feeding management.

### Overstocking and Cow Responses

A primary factor that influences feeding behavior and feed intake is stocking density. Overstocking is a common occurrence in the US dairy industry. A USDA-

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NAHMS survey of free-stall dairy farms reported that 58% of farms provided less than 0.60 m/cow of bunk space (i.e., current dairy industry recommendations for feeding space; NFACC, 2009) and 43% provided less than one stall per cow (USDA, 2010). In a survey of the northeastern US, feed bunk stocking density averaged 142% with a range of 58 to 228% (von Keyserlingk et al., 2012). The continued prevalence of overstocking reflects its association with maximizing profit per stall (De Vries et al., 2016).

Current economic analysis suggests that some degree of overstocking may be optimal if the focus is solely on profitability. De Vries et al. (2016) used published data to model the relationships among stocking density (stalls and feed bunk), lying time, and profit (\$/stall/year). This economic analysis reported that profit per stall actually was maximized around 120% stocking density for prevailing costs of production and milk price in the US. The profitability of overstocking was a function of revenue gained by increasing production per stall, the cost of increasing or decreasing production per cow, variable costs (i.e., costs that vary with changes in milk production), and milk price (De Vries et al., 2016). However, overstocking reduces the cow's ability to practice natural behaviors (Wechsler, 2007) which is a primary factor related to cow well-being.

Overstocking interferes with the cow's ability to practice normal feeding and resting behaviors, which comprise approximately 70% of the cow's day (Grant and Albright, 2001). Cows place priority on resting when forced to choose among resting, eating, and other behaviors (Metz, 1985; Munksgaard et al., 2005) which suggests that overstocking may limit their ability to meet their daily time budget, defined as 3 to 5 h/d of feeding, 10 to 14 h/d of lying, and 7 to 10 h/d of rumination (Grant and Albright, 2001; Gomez and Cook, 2010). Bach et al. (2008) were able to isolate the effect of management environment on cow performance using 47 dairy farms that were members of the same cooperative and fed the same TMR. Despite similar genetics and the same diet, average herd milk production ranged from 20.6 to 33.8 kg/d. The housing environment explained 56% of this variation and free stall stocking density accounted for 32% of the variation among farms by itself.

Higher stocking densities reduce feeding time and increase aggression at the feed bunk (Huzzey et al., 2006), may reduce rumination (Batchelder, 2000), decrease rumination while recumbent (Krawczel et al., 2012a), and reduce lying time (Fregonesi et al., 2007; Krawczel et al., 2012b). Overstocking also increases rate of feed consumption and meal size (Collings et al., 2011).

### Stocking Density as a Subclinical Stressor

The concept of subclinical stressors suggests that the summation of two stressors, such as housing and feeding management, will be greater than either in isolation. A subclinical stressor depletes the animal's biological resources without generating a detectable change in function, which leaves the animal without the resources to respond to subsequent stressors (Moberg, 2000). Therefore, subordinate animals may exhibit changes in behaviors that do not always result in clinical or visible outcomes such as lower milk production or altered health status. However, the subclinical stressor of



stocking density would diminish her effectiveness against additional stressors, placing her in a state of distress. Additional stressors are likely to occur due to constant changes in feeding and cow management.

### Experiment 1. Overstocking and Physically Effective Fiber

In our first study, forty-eight multiparous and 20 primiparous Holstein cows were assigned to 1 of 4 pens ( $n = 17$  cows per pen). Pens were assigned to treatments in a 4 x 4 Latin square with 14-d periods using a 2 x 2 factorial arrangement. Two stocking densities (**STKD**; 100 or 142%) and 2 diets (**straw, S** and **no straw, NS**; Table 1) resulted in 4 treatments (100NS, 100S, 142NS, and 142S). Stocking density was achieved through denial of access to both headlocks and free-stalls (100%, 17 free-stalls and headlocks per pen; 142%, 12 free-stalls and headlocks per pen). Pen served as the experimental unit.

Diets were similar except that the S diet had a portion of haycrop silage replaced with chopped wheat straw and soybean meal. Each diet was formulated to meet both ME and MP requirements. The TMR was mixed and delivered once daily at approximately 0600 h and pushed up approximately 6 times daily. The diets were designed to differ meaningfully in physically effective neutral detergent fiber (**peNDF**) and undigested NDF (**uNDF**) measured at 30, 120, and 240 h of *in vitro* fermentation. Otherwise, the two diets were similar in analyzed chemical composition.

Twelve multiparous and 4 primiparous ruminally fistulated cows were used to form 4 focal groups for ruminal fermentation data. Each focal group was balanced for DIM, milk yield, and parity. Ruminal pH was measured using an indwelling ruminal pH measurement system (Penner et al., 2006; LRCpH; Dascor, Escondido, CA) at 1-min intervals for 72 h on days 12, 13, and 14 of each period. Daily ruminal pH measurements were averaged over 10-min intervals. Measurements were then averaged across days and among cows into a pen average for each period.

Ruminal pH results are presented in Table 2. As expected, increasing the peNDF content of the diet reduced the time spent below pH 5.8 ( $P = 0.01$ ) as well as decreasing the severity of subacute ruminal acidosis (**SARA**) as observed through a reduction in area under the curve below pH 5.8 ( $P = 0.03$ ). Higher stocking density increased time spent below pH 5.8 ( $P < 0.01$ ) and tended to increase the severity of SARA ( $P = 0.06$ ).

Furthermore, there was a trend for an interaction between stocking density and diet, indicating greater SARA when cows were housed at higher stocking density and fed the lower fiber diet. Importantly, greater stocking density had a larger effect on ruminal pH than changes to the diet, with a 1.4-h difference between 100 and 142% stocking density but only a 0.9-h difference between diets. Reductions in SARA through the addition of straw was observed at both stocking densities (0.4-h difference at 100% and 1.4-h difference at 142%), although there seemed to be greater benefit of boosting dietary peNDF or uNDF at the higher stocking density.

Cows were milked 3 times daily and milk yields were recorded electronically on d 8 to 14 of each period. Milk samples were collected across 6 consecutive milkings for each cow on d 13 and 14 of each period and analyzed for composition. Ingestive, rumination, and lying behavior as well as the location (feed bunk, stall, alley, standing or lying) of these performed behaviors were assessed on all cows using 72-h direct observation at 10-min intervals (Mittlöhner et al., 2001) on d 8, 9, and 10 of each period.

Eating time (238 min/d, SEM = 4) and rumination time (493 min/d, SEM = 9) did not differ among treatments ( $P > 0.10$ ; Table 3). However, rumination within a free-stall as a percentage of total rumination decreased at higher stocking density. As resting and rumination are significant contributors to buffer production (Maekawa et al., 2002b), it is possible that this shift in the location of rumination may affect the volume or rate of buffer production, partially explaining the increased risk of SARA at higher stocking densities. Ruminal pH differences between diets are likely explained by increased buffer volume produced during eating and rumination for the straw diets as evidenced by Maekawa et al. (2002a) where increases in the fiber-to-concentrate ratio resulted in increased total daily saliva production.

Higher stocking density increased the latency to consume fresh feed – i.e., it took cows longer to approach the bunk and initiate eating with higher stocking density. Additionally, higher stocking density reduced lying time, but boosted the time spent lying while in a stall indicating greater stall-use efficiency. Overall, time spent standing in alleys increased markedly with overstocking.

There were no differences in DM intake among treatments, although as expected the straw diet increased both peNDF and uNDF<sub>om240</sub> intake. Changes in milk production were small, which would be expected given the short periods (14-d) used in this study (Table 4).

### Experiment 2. Overstocking and Reduced Feed Access

Nutrition models calculate nutrient requirements assuming that cows have ad libitum access to feed and are not overstocked. The reality is that the majority of cows in the US are fed under overstocked conditions – and increasingly farmers are feeding for lower amounts of daily feed refusals in an effort to minimize wastage of expensive feed. Consequently, we need to understand the interaction of stocking density and feed availability on ruminal pH, behavior, and productive efficiency.

Forty-eight multiparous and 20 primiparous Holstein cows were assigned to 1 of 4 pens ( $n = 17$  cows per pen). Pens were assigned to treatments in a 4 x 4 Latin square with 14-d periods using a 2 x 2 factorial arrangement. As in experiment 1, two STKD (100 or 142%) were used. In experiment 2, we evaluated 2 levels of feed restriction (0-h or no restriction; **NR**) and 5-h of feed restriction (**FR**) that resulted in 4 treatments (100NR, 100FR, 142NR, and 142FR). As in experiment 1, stocking density was achieved through denial of access to both headlocks and free-stalls (100%, 17 free-stalls and headlocks



per pen; 142%, 12 free-stalls and headlocks per pen) and pen served as the experimental unit.

Feed access was achieved through pulling feed away from headlocks approximately 5 h before the next feeding. Previous research has shown that blocking access to the feed bunk for 5 to 6 h/d mimics so-called "clean bunk" management (French et al., 2005). Sixteen multiparous ruminally fistulated cows were used to form 4 focal groups for ruminal fermentation data. Each focal group was balanced for days in milk, milk yield, and parity.

The effect of stocking density and feed access on ruminal pH characteristics is shown in Table 5. Higher stocking density, as in experiment 1, increased risk for SARA with greater time spent below pH 5.8 ( $P = 0.02$ ) and tended to increase severity ( $P = 0.09$ ). While there were no differences in ruminal pH responses for the feed access treatment, there was a significant interaction between stocking density and feed access ( $P = 0.02$ ), indicating an exacerbated risk for SARA when cows were housed at higher stocking density and had restricted access to feed. Compared to experiment 1, feed access when isolated did not have as great an impact on ruminal pH compared to differences in fiber levels of the diet. However, when combined with high stocking density, reduced feed access had a greater impact than the low fiber diets. The implications of these results on commercial dairy farms where overstocking and feeding to low levels of feed refusals is commonly practiced need to be better understood.

#### **Food for Thought. Re-Assessing Industry Feeding Management Norms**

**Competition for feed.** Cows have a naturally aggressive feeding drive and exert up to 226 kg of force against the feed barrier as they reach for feed (Hansen and Pallesen, 1998). To put this in perspective, 102 kg of force causes tissue bruising. Cows will injure themselves in an attempt to eat if we do not properly manage the feeding system to ensure feed accessibility. Even more importantly, a feeding environment that chronically frustrates a cow's drive to access feed may train her over time to become a less aggressive feeder (Grant and Albright, 2001).

Are 24 in (60 cm) of bunk space per cow - the industry standard - sufficient from the cow's perspective? A study by Rioja-Lang et al. (2012) addressed this question by providing subordinate cows with a choice: they could choose to eat a low palatability feed alone or they could choose a high palatability feed that came with a dominant cow located either 20, 45, 60, or 76 cm away. When feeding space was highly restrictive (i.e., 20 or 45 cm) most subordinate cows chose to eat the low palatability feed alone. But, even with 60 or 76 cm of feed space about 40% of subordinate cows still chose to eat alone. This research implies that some cows will settle for less desirable feed to avoid competition even when bunk space exceeds the current industry standard.

**Feeding frequency.** Delivery of fresh feed stimulates feeding behavior more than return from the parlor or feed push up. In a study that investigated herd-level management and milk production, Sova et al. (2013) found a benefit of twice over once

daily feeding with dry matter intake increasing 1.4 kg/d while milk yield increased by 2.0 kg/d. With 2x feeding of a TMR, more feed was available throughout the day and there was less feed sorting. Other research has found that greater feeding frequency of the TMR improves rumen fermentation, enhances rumination, and boosts eating time. The positive response to greater feeding frequency is more noticeable during heat stress conditions (Hart et al., 2014).

However, some research indicates that the positive response to greater feed delivery may diminish at high frequencies, such as 4 or 5 times per day (reviewed by Grant, 2015). In these cases, greater feeding frequency enhances eating time but also reduces resting time by up to 12%. Enhancements in feeding time should not be at the expense of time spent resting.

**Feed push-up.** Effective feed push-up strategy is critical for ensuring that feed is within easy reach of the cow and is a function of the number of times per day and when the feed push up occurs. A study conducted at the University of Arizona (Armstrong et al., 2008) evaluated the effect of feed push up each half-hour for the first two hours after feed delivery versus only once per hour.

Greater frequency of feed push up during the two hours after feed delivery resulted in more milk and improved efficiency with no impact on stall resting time (Table 6). The number of times that feed is pushed up throughout the day is important, but this research highlights the critical importance of timing of feed push up. When deciding a feed push up strategy, we need to focus on ensuring that feed is easily within reach of the cow during the highly competitive two hours following feed delivery.

**Feed refusals and availability.** For competitive feeding situations, each 2%-unit increase in feed refusals is associated with a 1.3% increase in sorting (Sova et al., 2013). Likewise, milk/DMI decreases by 3% for each 1% increase in sorting. Research has found little effect of feed refusal on efficiency of milk production over a fairly wide range of 2.5 to 16% refusals. On farm experience suggests that a refusal target of approximately 3% works well for lactation pens, but fresh pens should be closer to 6 or 7% to ensure that feed availability is never limiting.

How long can the feed bunk be empty? The cow's motivation to eat increases markedly after only 3 h without feed (Schutz et al., 2006). In addition, when feed access time is restricted by 10 hours per day, from 8:00 pm to 6:00 am, feed intake is reduced by 1.6 kg/d coinciding with twice as many displacements at feeding (Collings et al., 2011). When this temporal feed restriction is combined with overcrowding (1:1 or 2:1 cows per feeding bin) there is a 25% increase in feeding rate during the first 2 h after feed delivery (i.e., slug feeding).

#### **Conclusions**

Stocking density exhibited a consistent negative effect on ruminal pH and increased the risk for SARA. The presence of additional stressors in combination with



stocking density exacerbated these negative effects on ruminal pH, although the magnitude varied depending on the type of stressor. Manipulation of the feeding environment can help mitigate the negative effects of stocking density, such as increasing peNDF or uNDF240om in the diet or minimizing time without access to feed.

As new information is published we need to continually re-assess our feeding management recommendations. If we ask the cow for her opinion using well-designed studies and field observations, we will design optimal feeding environments. Recommended feeding management based on the latest research includes:

- Management that enhances rest and rumination
- Feed available on demand
- Consistent feed quality and quantity along the length of the bunk
- Bunk stocking density  $\leq 100\%$  ( $\geq 60$  cm/cow)
- Feed push up focused on 2 hours after feed delivery
- About 3% feed refusal target
- Bunk empty no more than 3 h/d (ideally never)

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**Table 1.** Ingredient composition and analyzed chemical composition (dry matter basis) of TMR samples for NS (No Straw) and S (Straw) experimental diets

	NS	S	SEM <sup>1</sup>
Ingredient, % of DM			
Conventional corn silage	39.72	39.73	
Haycrop silage	6.91	2.33	
Wheat straw, chopped	...	3.45	
Citrus pulp, dry	4.82	4.82	
Whole cottonseed, linted	3.45	3.45	
Soybean meal, 47.5% solvent	...	1.12	
Molasses	3.20	3.20	
Concentrate mix	41.89	41.88	
Chemical composition			
CP, % of DM	15.0	15.1	0.3
NDF, % of DM	30.8	30.1	0.4
Acid detergent lignin, % of DM	3.8	3.8	0.1
Starch, % of DM	25.0	25.5	0.5
Sugar, % of DM	7.4	8.1	0.4
Ether extract, % of DM	5.9	5.7	0.1
7-h starch digestibility, % of starch	73.3	74.3	0.9
Physically effective NDF <sub>1.18-mm</sub> , % of DM <sup>2</sup>	23.9	25.9	0.7
30-h uNDFom, % of DM <sup>3</sup>	13.1	14.9	0.3
120-h uNDFom, % of DM <sup>3</sup>	9.0	10.2	0.2
240-h uNDFom, % of DM <sup>3</sup>	8.5	9.7	0.2

<sup>1</sup> SEM = Standard error of the mean.

<sup>2</sup> peNDF determined with method described by Mertens (2002).

<sup>3</sup> Undigested NDF determined with method described by Tilley and Terry (1963) with modifications (Goering and Van Soest, 1970).

**Table 2.** Ruminal pH responses to diets containing straw (S) or no straw (NS) fed at 100 or 142% stocking density (STKD)

Variable	100%		142%		SEM <sup>1</sup>	P-value		
	NS	S	NS	S		STKD	Diet	STKD x Diet
Mean pH	6.17	6.13	6.09	6.10	0.03	0.07	0.62	0.39
Minimum pH	5.70	5.67	5.62	5.59	0.05	0.11	0.53	0.95
Maximum pH	6.63	6.58	6.56	6.53	0.04	0.07	0.22	0.68
Time pH < 5.8, h/d	2.29	1.90	4.12	2.77	0.41	<0.01	0.01	0.10
AUC < 5.8 pH <sup>2</sup>	0.38	0.19	0.58	0.34	0.10	0.06	0.03	0.75

<sup>1</sup> SEM = standard error of the mean.

<sup>2</sup> Area under the curve (pH x unit).



**Table 3.** Behavioral responses for cows fed diets containing straw (S) or no straw (NS) at 100 or 142% stocking density (STKD)

Item	100%		142%		SEM <sup>1</sup>	P-value		
	NS	S	NS	S		STKD	Diet	STKD x Diet
Eating time, min/d	233	237	242	240	4	0.13	0.76	0.48
Eating time/kg NDF, min	31.0	28.7	34.1	30.0	1.3	0.04	0.01	0.35
Eating time/kg peNDF, min	37.8	35.1	41.3	36.4	1.7	0.11	0.03	0.44
Eating, bouts/d	6.8	6.7	7.0	6.9	0.1	0.60	0.11	0.64
Meal length, min/meal	34.8	36.4	35.6	37.0	0.9	0.43	0.11	0.90
Eating latency fresh feed, min	20	28	39	40	4	0.02	0.35	0.46
Length of first meal, min	39	43	41	44	2	0.23	0.02	0.66
Rumination time								
Total, min/d	498	491	489	496	9.0	0.72	0.96	0.19
Per kg NDF, min	65.8	59.4	68.0	61.8	2.2	0.21	<0.0	0.95
Per kg peNDF, min	80.3	72.6	82.4	75.0	3.1	0.39	0.02	0.95
Ruminating in stall, % of total	86.2	86.0	80.5	81.1	<0.1	<0.01	0.96	0.60
Lying time, min/d	832	827	779	797	11	<0.01	0.56	0.31
Lying within stall, % of use	89.7	89.9	91.7	92.8	<0.01	0.01	0.39	0.50
Time in alley, min/d	121	125	192	181	9	<0.01	0.65	0.37

<sup>1</sup> SEM = standard error of the mean.

**Table 4.** Short term (14-d periods) feed intake and milk yield as influenced by stocking density (STKD) and diets containing straw (S) or no straw (NS)

Item	100%		142%		SEM <sup>1</sup>	P-value		
	NS	S	NS	S		STKD	Diet	STKD x Diet
Intake responses								
DM, kg/d	25.4	25.3	25.3	25.2	0.4	0.78	0.69	0.87
NDF, kg/d	7.5	8.3	7.2	8.0	0.3	0.23	<0.01	0.91
peNDF, kg/d	6.2	6.8	6.0	6.6	0.3	0.42	0.02	0.95
uNDF <sub>om240</sub> , kg/d	2.2	2.5	2.1	2.5	0.1	0.50	<0.01	0.22
Lactational responses								
Milk, kg/d	41.2	40.4	40.7	40.0	0.7	0.21	0.06	0.79
SCM, <sup>2</sup> kg/d	42.6	42.4	42.7	41.5	0.8	0.25	0.09	0.23

<sup>1</sup> SEM = standard error of the mean.

<sup>2</sup> SCM = solids-corrected milk.

**Table 5.** Ruminal pH responses as influenced by stocking density (STKD) and feed restriction (NR = no restriction; FR = 5 h restriction)

Item	100%		142%		SEM <sup>1</sup>	P-value		
	NR	FR	NR	FR		STKD	R <sup>2</sup>	STKD x R
Mean pH	5.96	6.03	5.98	5.89	0.06	0.14	0.80	0.08
Minimum pH	5.42	5.50	5.51	5.39	0.07	0.81	0.78	0.12
Maximum pH	6.49	6.61	6.48	6.53	0.04	0.25	0.06	0.29
Time pH < 5.8, h/d	6.62	5.23	6.78	8.77	1.27	0.02	0.49	0.02
AUC < 5.8 pH <sup>3</sup>	1.66	1.24	1.73	2.55	0.63	0.09	0.52	0.11

<sup>1</sup> SEM = standard error of the mean.

<sup>2</sup> R = effect of feed restriction (NR vs. FR).

<sup>3</sup> Area under the curve (pH x unit).

**Table 6.** Greater feed push up in hours after feed delivery improves efficiency

Cow response	1 time/hour	2 times/hour
Dry matter intake, kg/d	18.8	18.2
Milk, kg/d	27.9 <sup>b</sup>	29.7 <sup>a</sup>
Milk/dry matter intake, kg/kg	1.48 <sup>b</sup>	1.63 <sup>a</sup>
Lying in stall, % of cows	45.3	43.8

<sup>ab</sup> Means within row differ ( $P < 0.05$ ).



## SESSION NOTES

### Monensin Effects On Beef Cattle Grazing Warm-Season Perennial Grasses

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#### Introduction

Warm-season perennial grasses are the main forage used for beef cattle production in the southeastern USA (Ball et al. 1991). In general, forage-based cow-calf systems in tropical and subtropical regions are characterized by extensive grazing with low input levels.

Ionophores have been used to increase efficiency of ruminant production and monensin has been the most used ionophore in the US. Although the mechanisms are not completely elucidated, the main effects of monensin on ruminants are: 1) Shift in production of volatile fatty acids (**VFA**), 2) Change feed intake and digestibility, 3) Alter gas production, and 4) Increase protein use efficiency.

Monensin can be described as a cation-proton antiporter and mediates primarily  $\text{Na}^+/\text{H}^+$  exchange. The affinity of Monensin for  $\text{Na}^+$  is 10 times greater than that for  $\text{K}^+$  (Bergen and Bates, 1984). Accepted mechanisms by which ionophores negatively affect bacteria include non-physiological ion leak caused by ionophores and consequently adenosine triphosphate (**ATP**) depletion. This effect is greater in gram-positive bacteria. Gram-negative bacteria have a cellular envelope (outer membrane) that serves as a protective barrier, excluding ionophore complexes (Russell and Strobel, 1989).

Bergen and Bates (1984) summarized the effects of monensin as follow: the ionophore acts on the flux of ions through the membranes dissipating cation and proton gradients and interfering with the update of solutes and the primary transport system in the cells. The organisms try to maintain the transport by expending metabolic energy. The gram-negative bacteria can survive better to ionophore because they are able to produce ATP through the electron transport and there is a favorable shift of gram-negative bacteria in the rumen. Although Bergen and Bates (1984) postulated that monensin would cause entry of protons into ruminal bacteria in exchange for  $\text{Na}^+$ , Russell (1987) showed that the direction of  $\text{Na}^+$  was the opposite using *Streptococcus bovis* as a model. Monensin decreased intracellular  $\text{K}^+$  concentration and influx of protons, resulting in lower intracellular pH. Once intracellular pH was acidic, monensin produced an efflux of protons in exchange for  $\text{Na}^+$ . The inhibition of *S. bovis* was attributed to futile cycling of ions across the cell membrane resulting in loss of intracellular  $\text{K}^+$ , accumulation of intracellular  $\text{Na}^+$  and depletion of ATP.

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There have been several articles promoting the use of monensin in beef cattle on pasture with limited levels of supplementation. It is known that monensin select microorganisms in the rumen and enhance fermentation efficiency by increasing propionic acid and decreasing methane production. However, in extensive grazing systems, the animals are usually consuming limited amount of propionic acid precursors, such as starch and sugars, and therefore, it is expected that monensin may not be an efficient feed additive to be used under those conditions. Conversely, beef cattle supplemented on pasture with significant levels of concentrate may consume considerable amount of starch and sugars, which may enhance the effects of monensin and result in increased animal performance.

There are few published research studies reporting the effects of monensin on performance and forage intake of beef cattle grazing warm-season grass pastures. Feed efficiency data is rarely available because inherent difficulty in measurement of feed intake in grazing animals, therefore, there may be trials with no change in averaged daily gain (ADG) and decreased pasture intake; however, the forage intake was not measured or the scientific methods do not have precision to detect small variations in forage intake. In this case, the benefit of feeding an ionophore will be realized only if stocking rate is increased. Rouquette et al. (1980) compared the effects of monensin on beef calves grazing Bermudagrass and receiving 0.9 kg/d of concentrate. Calves receiving 200 mg monensin daily had greater ADG (0.54 vs. 0.40 kg/d) than calves receiving the concentrate only. It seems clear that monensin can improve the efficiency of utilization of concentrate supplements to cattle grazing warm-season grasses; however, the effect of ionophore to no supplemented animals is not consistent. Parrott et al. (1990) reported 8 trials of beef cattle grazing native warm-season grasses or Bermudagrass and observed variable responses of ADG to monensin and there was a trend for greater numerical ADG when the 8 trials were combined (0.90 vs. 0.93 kg/d). Walker et al. (1980) indicated that dry matter intake levels may be reduced by 5-10% when beef cows are supplemented with 200 to 300 mg/d of monensin. Randel and Rouquette (1976) reported that 200 mg/d monensin reduced dry matter intake of lactating beef cows by 12.4%; however, monensin did not affect milk production and composition. Clanton et al. (1981) fed 95, 90, and 90 % of the amount of forage consumed by control cows to beef cows fed 50, 200, or 300 mg/d of monensin. Cows fed 200 and 300 mg/d of monensin and 90% as much forage as control cows had similar weight gains as control cows.

Therefore, the effects of monensin on beef cattle receiving warm-season perennial grasses with limited supplementation is inconsistent. Research has been conducted at the University of Florida IFAS Range Cattle Research and Education Center to develop management practices to increase the efficiency of using monensin under those conditions.

#### Recent Research Conducted in Florida

Recent studies conducted at the IFAS Range Cattle Research and Education Center tested the effects of monensin (200 mg/d) to beef heifers grazing Bahiagrass (*Paspalum notatum*) pastures at two stocking rates, 1.6 or 2.5 heifers/ha for 2 yr in Florida

(Vendramini et al. 2015). Heifers received 0.4 kg of a concentrate supplement daily. The objective of the study was to verify the effectiveness of monensin in grazing animals with limited supplementation and forage quantity. Due to the seasonality of forage production in tropical areas, beef cattle are subject to limited forage allowance during some months of the year. Pastures grazed with greater stocking rates had lesser herbage mass (2,300 vs. 2,800 kg/hectare [ha]) and herbage allowance (HA, 1.0 vs. 1.8 kg DM/kg live weight, LW); however, there was no effect of stocking rates on forage crude protein (CP, 8.5%) and *in vitro* digestible organic matter (IVDOM, 49.7%). Pastures grazed by heifers receiving monensin or control had similar herbage mass, allowance, and nutritive value. There was a month by stocking rate interaction response on heifer ADG (Table 1); however, there was no effect of monensin supplementation on ADG (mean = 0.44 kg/d). According to Inyang et al. (2010), forage allowance below 1.4 kg DM/kg LW would limit performance of beef heifers grazing Bahiagrass pastures. The combinations of environmental factors and forage characteristics may have limited the potential positive effects of the monensin. The same authors quantified the effects of monensin (200 mg/d) on forage intake of heifers receiving Stargrass (*Cynodon nlemfuensis*) hay (11% CP, 51% IVDOM) and observed that there was no difference in total DM intake (2.1% body weight, BW) or forage DM intake (2.0% BW) between treatments.

Vendramini et al. (2015) evaluated the effects of increasing levels of monensin on rumen and blood metabolites of steers receiving Bermudagrass hay with limited supplementation. Treatments were 4 levels of monensin (0, 125, 250, or 375 mg monensin/d) added to a daily concentrate supplement fed at 0.2% BW. Considering a voluntary DM intake of 2.2% BW, these monensin levels were designed to supply the equivalent of 0, 10, 20, and 30 mg/kg/animal/d and create a wide range of doses, including the minimum and maximum doses recommended for grazing beef cattle, 50 and 200 mg/d, respectively. There was an increase in propionic acid concentration in the rumen and a tendency to decrease acetic acid. Rumen pH, ammonia, isobutyric, and butyric acid concentrations were not affected by treatments (Table 2). In addition, there was no effect on dry matter intake, 2.1 % BW. The slight increase in propionic acid was not sufficient to increase blood glucose, insulin-like growth factor-1 (IGF-1), and insulin concentrations. The increasing levels of monensin may have caused a change in microbial populations and the fermentation profile in the rumen, thus optimizing propionate formation. However, the magnitude of increase was insufficient to increase blood metabolites.

In a similar study, Moriel et al. (2018) tested the effects of adding 200 mg/d of monensin to molasses supplementation of beef heifers grazing Bahiagrass pastures during the summer and autumn in Florida. Heifers were offered 14 kg of sugarcane molasses and 3.5 kg of cottonseed meal weekly from day 0 to 84. Weekly supplement amount was divided and offered 3 times weekly on Monday, Wednesday, and Friday at 0800 h. Supplement DM disappearance (% of initial supplement DM offered) was determined every other week at 4, 10, 24, 28, and 34 h after morning supplementation. On d 85, heifers allocated to individual drylot pens, provided free choice access to Bermudagrass hay, and remained on their respective treatment for 10 d of adaptation and 11 d of data collection. The addition of monensin to the supplement did not impact



heifer BW, BCS, overall ADG, Bahiagrass IVDOM, CP, herbage mass, and herbage allowance from day 0 to 84 (Table 3). Supplement disappearance after 10 and 34 hours of supplementation was greater for control vs. monensin heifers and tended to be greater for control vs. monensin heifers 24 hours after supplementation (Table 4). Plasma concentrations of glucose, IGF-1, and BUN did not differ between treatments. During the drylot phase, forage DM intake, total DM intake, heifer BW and ADG did not differ between treatments. In summary, the addition of monensin into sugarcane molasses-based supplements decreased the rate of consumption of the supplement but did not impact ADG and blood parameters of heifers grazing warm-season grasses with limited nutritive value.

Early weaning is an effective management practice to increase the likelihood of rebreeding of first-calf beef heifers in the southeast USA. Arthington and Kalmbacher (2003) verified greater pregnancy rates in first-calf heifers whose calves were weaned at 3 months of age (94%) than those whose calves were weaned at normal age (65%). However, the practice of early weaning calves is still a challenge for beef cattle producers, in part because of few management options for the weaned calves. Mild winters in the southern USA allow producers to raise early-weaned calves on pastures of cool- or warm-season grasses with at least 1% BW supplementation (Vendramini et al., 2006; 2007). With greater levels of concentrate, it is likely that monensin would be an effective additive to add to supplementation of early-weaned calves.

Vendramini et al. (2018) conducted two experiments to evaluate the effects of concentrate amount and monensin inclusion on growth and physiological parameters of early-weaned beef calves consuming warm-season grasses in drylot and pastures. In both experiments, treatments consisted of two concentrate DM amount (1 or 2% BW) and two inclusion rates of monensin (0 or 20 mg of monensin/kg of total DM intake). In the drylot, early-weaned beef calves (initial age =  $90 \pm 13$  d; initial BW =  $83 \pm 12$  kg) were distributed in 12 drylot pens (4 calves/pen; 3 pens/treatment) and provided Stargrass hay (9% CP and 52% IVDOM) at amounts to ensure 10% DM refusals for 56 d. On pasture, early-weaned heifer calves (initial BW =  $171 \pm 15$  kg) were allocated into Bahiagrass pastures on a continuous and fixed stocking rate (1 ha and 3 heifers/pasture; 3 pastures/treatment). In both experiments, effects of monensin inclusion  $\times$  concentrate amount were not detected for any variable (Tables 5 and 6), but overall ADG and plasma IGF-1 concentrations were greater whereas fecal coccidia egg counts tended or were less for calves offered concentrate with vs. without monensin inclusion (Tables 5 and 6). Calves offered concentrate at 2% of BW had greater overall ADG. Herbage mass, *in vivo* apparent digestibility, total DMI and plasma concentrations of glucose and IGF-1, less forage DM intake, and no effects on fecal coccidia egg counts compared to calves offered concentrate at 1% of BW (Tables 5 and 6).

Vendramini and Arthington (2008) evaluated the supplementation of different levels of concentrate to early-weaned calves grazing dormant warm-season perennial grass pastures during the winter and concluded that it was necessary 2% BW supplementation for calves to have a similar performance to the contemporaneous calves that were not early-weaned. The coccidiostat effect of monensin was an attractive

characteristic to potentially increase the performance of these early-weaned calves during the winter. Vendramini et al. (data not published) tested the effects of adding 20 ppm of monensin to the supplement of early weaned calves grazing dormant Bahiagrass in the winter and receiving 2% BW supplementation. The addition of monensin in the supplement resulted in significant increase in ADG from 0.8 to 0.9 kg/d. In addition, calves receiving monensin had 76% reduction in the incidence of coccidia. There was no difference in forage mass, implying that forage intake was similar among treatments. The calves were moved to a drylot and maintained in the same treatment for 30 d to evaluate the effect of monensin on DM intake. There was no effect of monensin on forage DM intake (0.7% BW) or total DM intake (2.6% BW).

## Conclusions

It was concluded that the positive effects of monensin on rumen fermentation and VFA proportion may be minimized due to the lack of substrate for propionate production in cattle receiving predominantly warm-season forages with limited concentrate supplementation. However, the addition of monensin may decrease supplement intake rate and be desirable in systems with infrequent supplementation on pasture.

Monensin should be supplied to early-weaned calves grazing warm-season pastures and receiving concentrate at 1% of BW or above because of the benefits in controlling coccidia and additional average daily gain.

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**Table 1.** Herbage mass and allowance, and average daily gain of heifers grazed on Bahiagrass pastures at different stocking rates (1.2 vs. 1.7 AU/ha)<sup>1</sup>

Response variable / stocking rate	Month				SE <sup>2</sup>
	June	July	August	September	
Herbage mass, kg/ha					
1.2 AU/ha	1,600 <sup>b</sup>	1,700 <sup>b</sup>	2,600 <sup>a</sup>	2,700 <sup>a</sup>	300
1.7 AU/ha	1,490 <sup>b</sup>	1,530 <sup>b</sup>	2,080 <sup>a</sup>	2,090 <sup>a</sup>	
P value <sup>3</sup>	0.20	0.18	< 0.01	< 0.01	
SE			450		
Herbage allowance, kg DM/kg BW					
1.2 AU/ha	1.1 <sup>b</sup>	1.3 <sup>b</sup>	2.3 <sup>a</sup>	2.3 <sup>a</sup>	0.1
1.7 AU/ha	0.9 <sup>b</sup>	1.0 <sup>b</sup>	1.3 <sup>a</sup>	1.4 <sup>a</sup>	
P value <sup>3</sup>	0.26	0.02	< 0.01	< 0.01	
SE			0.1		
Average daily gain (kg/d)					
1.2 AU/ha		0.3 <sup>b</sup>	0.6 <sup>a</sup>	0.6 <sup>a</sup>	0.1
1.7 AU/ha		0.1 <sup>b</sup>	0.3 <sup>b</sup>	0.6 <sup>a</sup>	
P value <sup>3</sup>		0.15	< 0.01	0.85	
SE			0.1		

<sup>a,b</sup> Within a row, means without a common superscript differ ( $P \leq 0.05$ ).

<sup>1</sup> AU/ha = animal units per hectare.

<sup>2</sup> SE = standard error.

<sup>3</sup> P value for the comparison of means between stocking rate treatments within month.

Data from Vendramini et al. (2015).

**Table 2.** Effects of supplemental levels of monensin on ruminal fermentation parameters of steers receiving Stargrass (*Cynodon nlemfuensis*) hay and supplemented with 0.2% body weight of concentrate

Item	Monensin (mg/kg)				Orthogonal contrast			SE <sup>1</sup>
	0	10	20	30	L	Q	C	
Ruminal pH	6.6	6.6	6.7	6.5	0.41	0.19	0.33	0.07
Propionate, mol/100 mol	16.9	17.9	19.1	19.4	0.004	0.56	0.64	0.5
Acetate, mol/100mol	74.0	73.1	71.3	71.1	0.09	0.91	0.82	1.0
Butyrate, mol/100 mol	8.7	8.4	8.3	8.5	0.82	0.72	0.98	0.7
Acetate:Propionate	4.3	4.0	3.7	3.6	0.001	0.19	0.65	0.2
Ammonia-N, mg/100 ml	7.3	6.1	6.4	7.3	0.86	0.17	0.79	0.7

<sup>1</sup> SE = standard error.

Data from Vendramini et al. (2015)



**Table 3.** Overall herbage mass (HM), herbage allowance (HA), in vitro organic matter digestibility (IVOMD) and crude protein (CP) of Bahiagrass pastures, and growth performance of heifers offered 14 kg of sugarcane molasses + 3.5 kg of cottonseed meal per heifer weekly (DM basis) from day 0 to 84

Item	Treatment (TRT)			P-value		
	Control	Monensin	SEM <sup>1</sup>	TRT	TRT x day	Day
HM, kg DM/ha	3,061	3,128	28.9	0.13	0.19	<0.0001
HA, kg DM/kg BW	4.37	4.19	0.273	0.65	0.36	<0.0001
IVOMD, %	40.3	41.2	0.75	0.46	0.80	0.0007
CP, % of DM	7.64	7.98	0.239	0.34	0.78	0.24
Body weight, kg						
d 28	363	360	3.6	0.63	0.66	<0.0001
d 56	368	363	5.4	---	---	---
d 84	388	386	6.0	---	---	---
Body condition day 84	6.34	6.37	0.152	0.89	---	---
ADG 0 to 84 d, kg/day	-0.04	-0.05	0.060	0.94	---	---
Body condition change	0.14	0.23	0.129	0.66	---	---

<sup>1</sup> SEM = standard error of the mean.

Data from Moriel et al. (2018).

**Table 4.** Supplement DM disappearance (% of initial DM offer) pattern of heifers offered 14 kg of sugarcane molasses + 3.5 kg of cottonseed meal per heifer weekly (DM basis) from day 0 to 84

Item	Treatment		SEM <sup>1</sup>	P-value
	Control	Monensin		
Supplement DM disappearance, % of initial offer				
4 hours	25.4	20.9	0.02	0.13
10 hours	81.8	75.5		0.04
24 hours	92.6	87.3		0.07
28 hours	96.7	92.5		0.16
34 hours	99.3	93.0		0.04

<sup>1</sup> SEM = standard error of the mean.

Data from Moriel et al. (2018).

**Table 5.** Responses of early-weaned calves offered free choice access to long-stem Stargrass hay in drylot and provided, in a 2 x 2 factorial arrangement, two amounts of concentrate DM (1 and 2% of body weight, BW) with or without monensin (20 mg/kg of total DM intake) for 56 d

Item	P-value			
	Concentrate		Monensin	
	1% BW	2% BW	0 mg/kg	20 mg/kg
Average daily gain, kg/d				
d 0 to 28	0.33	0.56	0.33	0.56
d 28 to 56	0.18	0.54	0.32	0.40
d 0 to 56	0.26	0.55	0.36	0.44
Plasma urea N, mg/dL	12.5	13.0	13.3	12.2
Plasma glucose, mg/dL	73.5	81.7	76.6	78.6
Plasma IGF-1, <sup>2</sup> ng/mL	39.7	59.7	46.2	53.0
Coccidia egg count on d 56 <sup>3</sup>	1.16	1.06	1.39	0.82
Forage DM intake, % body weight	1.5	0.9	1.2	1.2
Total DMI, % of body weight	2.3	2.6	2.5	2.5
In vivo apparent digestibility, %	56	62	58	60

<sup>1</sup> SEM = standard error of the mean.

<sup>2</sup> Insulin-like growth factor-1.

<sup>3</sup> Log<sub>10</sub> egg count/g of feces.

Data from Vendramini et al. (2018).



**Table 6.** Responses of early-weaned heifers grazing Bahiagrass pastures (3 heifers/pasture) and provided, in a 2 x 2 factorial arrangement, two concentrate DM amounts (1 and 2% of body weight, BW) with or without monensin (20 mg/kg of total DM intake) for 84 d

Item	Concentrate			Monensin			P-value	
	1% BW	2% BW	0 mg/kg	20 mg/kg	SEM <sup>1</sup>	Concentrate	Monensin	Concentrate x Monensin
Average daily gain, kg/d								
d 0 to 28	0.99	1.04	0.93	1.10	0.045	0.46	0.03	0.46
d 28 to 56	0.85	1.21	0.95	1.12	0.080	0.01	0.16	0.72
d 56 to 84	0.69	0.92	0.73	0.88	0.047	<0.01	0.05	0.14
d 0 to 84	0.85	1.06	0.87	1.03	0.042	<0.01	0.02	0.52
Plasma urea N, mg/dL	20.8	21.3	21.5	20.6	0.71	0.57	0.42	0.66
Plasma glucose, mg/dL	71.9	75.8	72.3	75.3	1.80	0.19	0.31	0.43
Plasma insulin, $\mu$ U/mL	2.02	2.81	2.25	2.58	0.519	0.31	0.67	0.60
Plasma IGF-1, <sup>2</sup> ng/mL	155	171	149	176	6.7	0.14	<0.01	0.30
Coccidia egg count on d 84 <sup>3</sup>	0.45	0.39	0.70	0.14	0.192	0.83	0.05	0.22
Herbage mass, kg/ha	3,700	4,400	4,100	4,100	200	0.09	0.75	0.71
Herbage allowance, kg DM/kg BW	8.0	10.0	9.4	9.6	0.4	0.06	0.69	0.90
CP, %	14.0	14.0	14.7	13.9	0.3	0.25	0.17	0.65
In vitro OM digestibility, %	48.5	48.8	48.5	48.9	0.52	0.64	0.59	0.78

<sup>1</sup> SEM = standard error of the mean.

<sup>2</sup> Insulin-like growth factor 1.

<sup>3</sup> Log<sub>10</sub> egg count/g of feces.

Data from Vendramini et al. (2018).

## SESSION NOTES



# Nutritional and Management Considerations to Minimize Stress and Optimize Production Efficiency in Cow-Calf Systems

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## Introduction

Stress response is defined as the reaction(s) of an animal to internal and external factors that influence its homeostasis and wellbeing (Moberg, 2000), whereas animals unable to cope with these factors are classified as stressed (Dobson and Smith, 2000). Within beef production systems, cattle are inevitably exposed to stress during their productive lives (Carroll and Forsberg, 2007), including psychologic, physiologic, and physical stressors associated with routine management practices (Cooke, 2017). In cow-calf systems, stressors may emerge from housing management, dietary and environmental changes, inadequate or excessive nutrition, disease, and cattle disposition. Hence, management to prevent and/or alleviate stressors is critical for optimal productive efficiency in cow-calf and beef production enterprises.

Although the physiologic consequences of stress are still not fully elucidated (Pacak and Palkovits, 2001), it has been demonstrated that stressors impact the immune system, as well as different responses within the body, mainly via the hypothalamic-pituitary-adrenal (HPA) axis (Elenkov, et al., 2000). Elevated cortisol is one of the main outcomes of the HPA reaction, independently if the stressor is from psychological, physiological, or physical nature (Cooke, 2017). This is the reason to why cortisol is generally considered the paramount to the neuroendocrine stress response (Sapolsky et al., 2000), and a major link between stress and productive functions (Cooke, 2017). Despite playing crucial roles in several body functions, cortisol degrades muscle and adipose tissues to increase the availability of energy to the animal. Cortisol has also been shown to impair physiological reactions associated with reproduction (Dobson et al., 2001). Supporting the negative impacts of stress + HPA axis in beef production systems, our group demonstrated a negative relationship between plasma cortisol concentrations and reproductive performance in beef females (Figure 1; Cooke, 2014, Cooke et al., 2017; Cooke et al., 2018).

## Stocking Density

High stocking density is perceived as a major stressor by livestock (Grandin, 2014). However, stocking density has been overlooked by US cow-calf producers due to the extensive nature of these operations (Asem-Hiablie et al., 2016). Yet, there are

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specific segments within cow-calf production where cattle are exposed to intensive management and housing, particularly replacement heifers. In typical US spring-calving herds ( $\geq 70\%$  of the nation's cow-calf operations; NASS, 2016), replacement heifers are weaned in the fall (~7 months of age) and exposed to their first breeding season the following spring (~15 months of age). During late fall and winter, heifers may be reared in drylot systems to ensure adequate feeding for growth (Olson et al., 1992; NASS, 2016) without specific considerations for stocking density. Moreover, intensifying cow-calf production by placing beef females in drylots during most or all of the year has been gaining attention (Lardy et al., 2017), as availability of grazing areas becomes limited by environmental challenges (e.g. drought), conversion to crop grounds, and use for non-agricultural purposes (e.g. accommodate urban expansion).

Despite the increasing number of beef females reared in drylots within the US cow-calf industry, our research group (Schubach et al., 2017) was the first to investigate and portray the potential adversities resultant from this management scheme to heifer welfare and reproductive development. We compared growth, physical activity, stress-related and physiological responses, and puberty attainment in beef heifers reared on high (14 m<sup>2</sup>/heifer, drylot; HIDENS) or low (25,000 m<sup>2</sup>/heifer, paddocks; LOWDENS) stocking densities from weaning until their first breeding season. The HIDENS was designed within the recommended stocking density for growing cattle reared in drylots (FASS, 2010). Heifers from both treatments received similar dietary regimens given that paddocks had no forage available for grazing, and a variety of stress-related, physical activity, and developmental responses were evaluated.

**Physical activity:** Heifers from HIDENS took fewer steps/week compared with LOWDENS (Table 1), given the larger area that LOWDENS heifers had available for movement. Hence, high stocking density reduced the opportunity for heifers to exercise.

**Physiological responses.** Cortisol concentration in hair from the tail switch is a validated biomarker of chronic stress in cattle (Burnett et al., 2014; Moya et al., 2015), given that cortisol is gradually accumulated in the emerging tail hair and its concentration represents long-term adrenocortical activity (Moya et al., 2013). Accordingly, cortisol concentration in hair from the tail switch were greater in HIDENS compared with LOWDENS heifers during the majority of the experimental period (Figure 2), corroborating that high stocking density chronically stimulated heifer adrenocortical activity. Mean expression of heat shock protein (HSP) 70 and HSP72 mRNA in whole blood during the experiment were greater in LOWDENS vs. HIDENS heifers (Table 1). Although HSP mRNA expression can also be used as diagnostic marker of stress, exercise upregulates and increases circulating concentrations of these HSP (Milne and Noble, 2002). Exercise activates the HSP response via several mechanisms including increased muscle temperature, exercise-related production of reactive oxygen species, and muscle ATP depletion (Noble et al., 2008). Hence, treatment effects detected for whole blood mRNA expression of HSP70 and HSP72 were also associated with the increased physical activity of LOWDENS heifers throughout the experiment, including prior to and during handling for sampling.



**Growth responses:** Elevated physical activity increases maintenance requirements in cattle (NRC, 2000). According to physical activity and space available to LOWDENS, their maintenance energy requirements were estimated to be 15% greater compared with that of HIDENS heifers (NRC, 2000). However, LOWDENS and HIDENS heifers had similar body weight (BW) gain during the experiment (Table 1), despite receiving the same diets and calculated differences in nutritional needs. Alternatively, the chronic stress experienced by HIDENS heifers likely increased their basal metabolism and maintenance requirements to the same level that physical activity increased these parameters in LOWDENS heifers.

**Reproductive development:** Puberty attainment was delayed in HIDENS compared with LOWDENS heifers (Table 1; Figure 3). Within heifers that reached puberty during the experiment, HIDENS were heavier and older at puberty compared with LOWDENS heifers (Table 1). Although age at puberty in cattle is highly determined by BW and growth rate (Schillo et al., 1992), high stocking density hindered puberty attainment despite similar growth between HIDENS and LOWDENS heifers. It is also important to note that heifers from both treatments achieved the recommended BW for puberty attainment during the experimental period (60-65% of mature BW; Patterson et al., 2000).

Results from Schubach et al. (2017) were novel and indicate that rearing heifers in drylots with a high stocking density is detrimental to welfare aspects including physical activity and chronic stress, resulting in delayed puberty despite adequate age and body development. Puberty attainment defines reproductive development, and regulates lifetime reproductive efficiency of beef females (Schillo et al., 1992). In turn, physical activity modulates circulating concentrations of endogenous opioids (Harber and Sutton, 1984), which impact secretion of gonadotropins required for a successful ovulation and puberty achievement in cattle (Mahmoud et al., 1989). Chronic stress and resultant increase in adrenocortical activity also impair gonadotrophin synthesis and reduce the sensitivity of the brain to estrogen (Dobson and Smith, 2000). Therefore, Schubach et al. (2017) exposed the need for research to investigate management and stocking density guidelines for beef heifers reared in drylots, which will contribute to enhancing welfare conditions and overall efficiency in US cow-calf systems.

### Stress from Change in Environment and Diet

Grazing and dietary habits are learned early in life, resulting in motor skills necessary to harvest and ingest forages (Provenza and Balph, 1987). Moreover, such skills learned between weaning and breeding have been reported to carry through to the next grazing season (Olson et al., 1992). Young ruminants consume small amounts of novel food and gradually increase the amount ingested if no adverse effects occur (Chapple and Lynch, 1986). Hence, replacement heifers often spend more time and energy foraging while ingesting less food when introduced to novel environments and feed sources (Osuji, 1974; Curl and Davidson, 1983). Accordingly, heifers that grazed forage from weaning to breeding rather than being placed in drylots retained better grazing skills and had increased average daily gains into the subsequent grazing season (Olson et al., 1992).

Following this rationale, Perry et al. (2013) compared BW change and pregnancy rates to artificial insemination (AI) in replacement beef heifers that were weaned into drylots and moved to pastures after breeding, compared with cohorts that were maintained on pasture since breeding. These authors reported less BW gain after AI in heifers originated from drylots, as well as reduced pregnancy rates to AI compared with those with previous grazing experience (Table 2). Hence, the stressors elicited by change in environment, associated with inadequate forage intake, contributed to decreased reproductive efficiency in drylot heifers moved to pastures upon AI (Perry et al., 2013).

### Excitable Temperament Also Is a Stressor

As mentioned above, stress response is defined as the reaction of an animal to internal and external factors that influence its homeostasis, and cattle unable to cope with these factors are classified as stressed (Dobson and Smith, 2000; Moberg, 2000). Based on this concept, the fearful and/or aggressive responses expressed by excitable cattle during human handling can be attributed to their inability to cope with this situation; therefore, classified as a stress response. Accordingly, excitable cattle typically experience changes in their neuroendocrine system and HPA axis that culminates with increased synthesis of cortisol. Several research studies reported that cattle with excitable temperaments have greater circulating cortisol concentrations during handling compared to cohorts with adequate temperament (Cooke, 2014). It is worth mentioning that the aforementioned studies evaluated *B. taurus*- and *B. indicus*-influenced cattle from different ages, genders, and across intensive and extensive systems. Hence, excitable temperaments have been positively associated with neuroendocrine stress reactions independent of breed type, age category, and production system.

As an initial attempt to associate temperament and reproduction in beef females, Plasse et al. (1970) classified *B. indicus* heifers according to temperament score (1 = calm, 2 = moderate, and 3 = excitable temperament) and reproductive score (heifers with inadequate reproductive performance received the greatest scores). These authors reported that temperament score was positively correlated with reproductive scores and negatively correlated with length of estrus, and suggested that consideration of temperament in selection programs might have a positive influence on the reproductive efficiency of the cowherd. However, the practical effects of excitable temperament on reproductive function of beef females still needed further investigation. Hence, our research group recently assessed the impacts of temperament on reproductive performance of *B. taurus* and *B. indicus*-influenced cows (Cooke et al., 2009; Cooke et al., 2011; Cooke et al., 2012).

Cooke et al. (2009) evaluated temperament at the beginning of the breeding season in Braford cows exposed to a 90-d bull breeding, and Brahman x British cows assigned to fixed-time AI followed by a 90-d bull breeding. Probability of pregnancy during the breeding season was negatively associated with temperament score, independently of breed and reproductive management (Figure 4). Similarly, Cooke et al. (2011) evaluated temperament in Nelore cows assigned to a fixed-time AI protocol, and reported



that cows with excitable temperament had reduced pregnancy rates compared to cohorts with adequate temperament (Table 3). More recently, Cooke et al. (2012) evaluated temperament at the beginning of the breeding season in Angus × Hereford cows assigned to 50-d bull breeding only, or fixed-time AI followed by a 50-d bull breeding. Cows with excitable temperament had reduced pregnancy rate, calving rate, weaning rate, and kg of calf weaned/cow exposed compared to cows with adequate temperament (Table 3), indicating that excitable temperament not only impairs reproductive performance, but also overall production efficiency in cow-calf systems.

Collectively, these results demonstrated that cows with excitable temperament had reduced reproductive performance compared to cohorts with adequate temperament. Such outcomes were independent of breed type (*B. taurus* and *B. indicus*-influenced cattle), reproductive management (AI, natural breeding, or both), and perhaps nutritional status because cow BCS at the beginning of the breeding season was not affected by temperament (Cooke et al., 2009; 2011; 2012). Plasma cortisol concentrations were greater in cows with excitable temperament (Cooke et al., 2009; 2012), which indicates that their decreased pregnancy rates could be attributed to neuroendocrine stress responses stimulated by handling for estrus synchronization and AI (Dobson et al., 2001). However, the same decrease in reproductive performance was observed in excitable cows assigned to natural breeding only, with no human interaction or handling to stimulate neuroendocrine stress responses during the breeding season. Therefore, additional mechanisms associating temperament and reproduction in beef females, including post-conception effects and potential genetic and innate deficiencies within the reproductive system of excitable cows, warrant further investigation (Cooke et al., 2012).

### Conclusions

Stress has direct implications to beef cattle production systems, including reproductive efficiency of beef females within cow-calf operations. These impacts are mediated, at least partially, by neuroendocrine stress reactions that hinder ovulation and pregnancy success. Moreover, stressors from different natures (physical, physiological, and psychological) stimulate similar negative responses to cattle welfare and production. Many of these stressors are elicited by routine production practices including stocking density, nutrition, transport, and cattle responses to human handling. Therefore, management that prevents or mitigate these stressors are warranted for optimal production efficiency of cow-calf operations.

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**Table 1.** Physical and physiological responses in beef heifers reared in low stocking density (25,000 m<sup>2</sup>/heifer; LOWDENS) or high stocking density (14 m<sup>2</sup>/heifer; HIDENS)

Item	LOWDENS	HIDENS	P-value
Physical activity, steps/week	19,709	3,148	< 0.01
Growth parameters			
Initial weight, kg	211	212	0.82
Final weight, kg	356	358	0.84
Growth rate, kg/day	0.777	0.783	0.82
HSP mRNA expression			
HSP70, fold effect	3.72	2.39	0.09
HSP72, fold effect	3.48	2.77	0.04
Puberty attainment			
Total pubertal heifers, %	65.4	31.9	< 0.01
Age at puberty, days	331	364	0.04
BW at puberty, kg	324	372	< 0.01

Adapted from Schubach et al. (2017).

**Table 2.** Reproductive performance of heifers that were weaned and developed on pasture compared to heifers weaned and developed in a drylot. All heifers were moved to pasture following artificial insemination (AI)

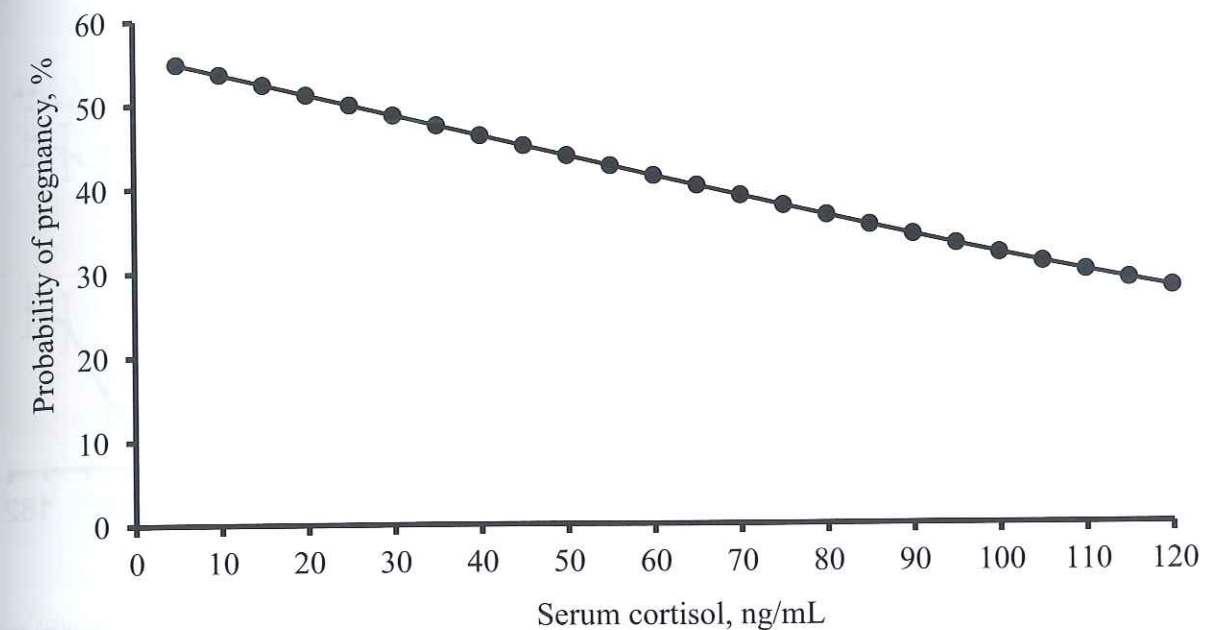
Item	Pasture	Drylot	P-value
Number of heifers	207	2014	--
Puberty status at AI, %	93.6	97.3	0.93
BW gain after AI, kg	0.94	0.13	< 0.01

Adapted from Perry et al. (2013).

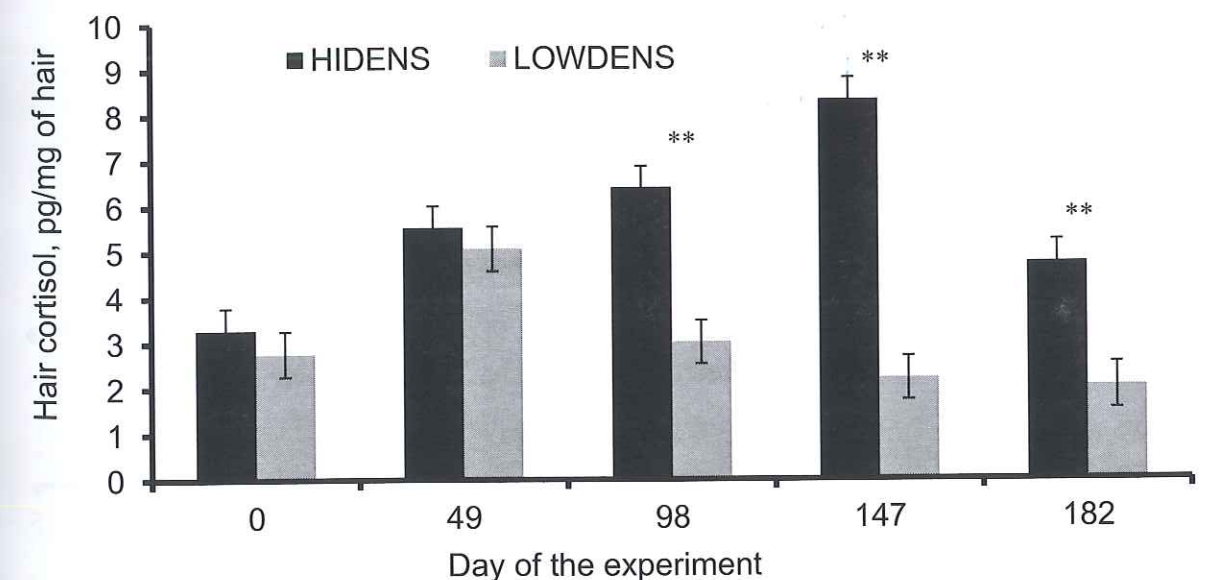
**Table 3.** Reproductive performance of beef cows according to temperament

Item	Adequate	Excitable	P-value
<i>Bos indicus</i>			
Pregnancy rate to AI, %	42.8	35.3	0.05
<i>B. taurus</i>			
Pregnancy rate (breeding season), %	94.6	88.7	0.03
Calving rate, %	91.8	85.0	0.04
Weaning rate, %	89.9	83.9	0.09
Calf weaning BW, kg	248	247	0.71
Calf BW weaned/cow exposed, kg	223	207	0.08

Adapted from Cooke et al. (2014).



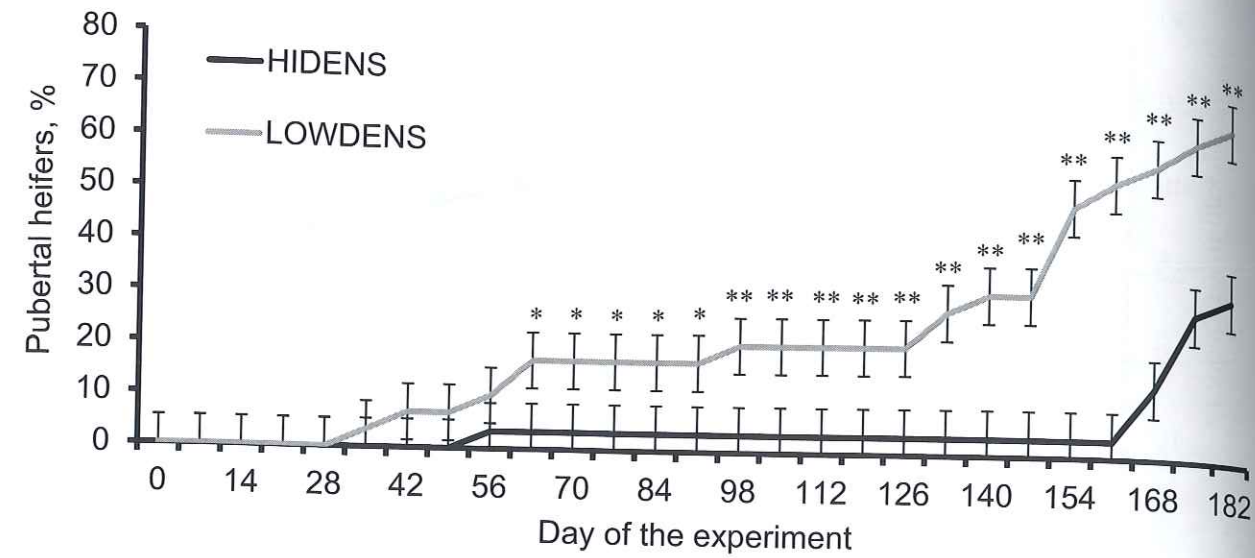
**Figure 1.** Probability of pregnancy to fixed-time artificial insemination (AI) in beef cows according serum cortisol concentrations at the time of AI. Pregnancy status was verified 30 d after AI via transrectal ultrasonography. A linear effect was detected ( $P < 0.01$ ). Adapted from Cooke et al. (2017)



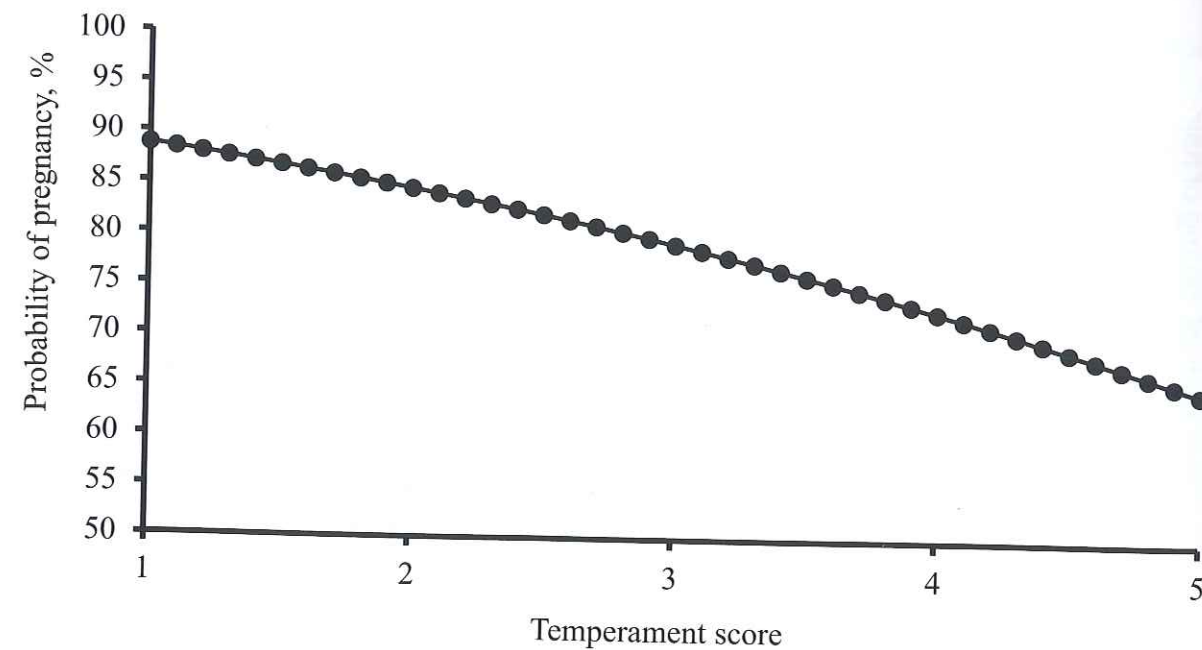
**Figure 2.** Cortisol concentrations in tail switch hair from heifers reared in low (25,000 m<sup>2</sup>/heifer; LOWDENS) or high stocking density (14 m<sup>2</sup>/heifer; HIDENS). \*\*  $P < 0.01$  and \*  $P \leq 0.05$ . Adapted from Schubach et al. (2017).



## SESSION NOTES



**Figure 3.** Puberty attainment in heifers reared in low stocking density (25,000 m<sup>2</sup>/heifer; LOWDENS) or high stocking density (14 m<sup>2</sup>/heifer; HIDENS). Within days, \*  $P \leq 0.05$  and \*\*  $P \leq 0.01$ . Adapted from Schubach et al. (2017).



**Figure 4.** Probability of pregnancy in beef cows according temperament score (1 = calm, 5 = excitable) at the beginning of the breeding season. A linear effect was detected ( $P < 0.01$ ). Adapted from Cooke et al. (2009).



## The Use of Milk Fatty Acids as an Indication of Energy Balance in Dairy Cows

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### Energy Balance and Changes in Milk Fatty Acid Composition

It is well known that in ruminant milk production, a significant amount of milk fatty (FA) acid are synthesized in the mammary cells (called the *de novo* FA) from  $\beta$ -hydroxybutyrate and acetate that are produced in the rumen as result of fermentation of dietary carbohydrates in the rumen (Palmquist et al., 1993). The  $\beta$ -hydroxybutyrate and acetate are carried to mammary cells through the blood stream and those substrates, Lynch et al. (1992) and Palmquist et al. (1993) showed that milk FA composition changed with stage of lactation with preformed milk FA being high during early lactation when cows were in negative energy balance and mobilizing body fat (i.e., long chain preformed FA), while synthesis of *de novo* FA was low. This relationship gradually change with increasing days in milk with the relative proportion of *de novo* FA increasing and the proportion of preformed FA decreasing with increasing days in milk (Lynch et al., 1992). In 2014, Barbano et al. (2014) reported a rapid mid-infrared (MIR) milk analysis method to measure both the concentration of *de novo*, mixed, and preformed milk FA in gram per 100 grams of milk and the relative portion of these groups of FA as a percentage of the total FA in milk fat. In addition, MIR prediction models were developed to measure average milk FA chain length expressed as carbon number per FA, total unsaturation expressed as double bonds per FA, and other individual milk FA plus milk estimated blood nonesterified FA (NEFA) value.

This MIR method could be used to rapidly analyze bulk tank, pen, and individual cow milks samples as a tool for nutrition and health management in dairy cows at all three levels. Currently, different models of MIR instruments (Delta Instrument, Drachten, The Netherlands) are available that can measure all of these parameters at a rate from 30 to 600 milk samples per hour. The application of MIR to measure *de novo*, mixed and preformed milk FA for dairy cattle feeding management was done for analysis of bulk tank milk samples because milk from every farm is tested a high frequency for milk payment testing and the milk FA analysis can be determined with the same instrument at the same time the milk payment test is being done on the same milk sample. This provides feed back to dairy farmers and nutritionists that reflects changes in the nutrition and health status of the complete dairy herd.

In 2014, Barbano et al. (2014) introduced the application of MIR for rapid milk FA analysis and reported positive correlations of bulk tank milk fat test with a higher proportion and concentration of *de novo* FA in bulk tank milk. The analytical aspects of

reference milk FA analysis and model development and validation were reported by Wojciechowski and Barbano (2016) and Woolpert et al. (2016). The form of the FA data from the MIR was structured to provide information on the relative proportions of *de novo* (C4 to C15), mixed origin (C16:0, C16:1, C17:0), and preformed (C18:0 and longer) FA in milk, the mean FA chain length (carbon number) and degree of unsaturation (double bonds/FA). With experience in the field testing milk from bulk tank milk from individual on farms we found that providing this FA information in units of grams per 100 grams of milk was more useful. Since that time, we have continued to collect data on milk FA variation in bulk tank milk and it's relationship to feeding and farm management.

Woolpert et al. (2016; 2017) have reported the results of two studies to determine feeding and farm management factors influencing milk FA composition and their relationship to bulk tank milk fat and protein test and production per cow per day of fat and protein. In the first study (Woolpert et al., 2016) with 44 commercial dairies that were identified as either predominantly Holstein or Jersey in northern Vermont and New York. The yields of milk fat, true protein, and *de novo* FA per cow per day were higher for high *de novo* (HDN) versus low *de novo* (LDN) farms. The HDN farms had lower freestall stocking density (cows/stall) than LDN farms. Additionally, tiestall feeding frequency was higher for HDN than LDN farms. No differences between HDN and LDN farms were detected for dietary dry matter, crude protein, neutral detergent fiber, starch, or percentage of forage in the diet. However, dietary ether extract was lower for HDN than LDN farms. Overall, overcrowded freestalls, reduced feeding frequency, and greater dietary ether extract content were associated with lower *de novo* FA synthesis and reduced milk fat and true protein yields on commercial dairy farms in this study.

The difference in income per cow would depend on the actual milk price at any point in time. The average fat and protein price for the Federal Milk Order No. 1 for March and April 2014 was \$4.62 and \$10.17 per kg (\$2.10 and \$4.62 per lb), respectively. Therefore, at 55 lb (25 kg) of milk per cow per day, the average HDN farm earned a gross of \$5.50 and \$7.72 per cow for fat and protein, respectively. The average LDN farm at 55 lb (25 kg) milk per cow per day earned a gross of \$5.26 and \$7.29 per cow for fat and protein, respectively. These differences for fat and protein between HDN and LDN herds at 55 lb (25 kg) of milk per 100 cows per year would result in a gross income difference of \$8,544 for fat and \$15,695 for protein.

A second study (Woolpert et al., 2017) with 39 commercial Holstein herds was conducted as a follow up to the previous study. No differences in milk (about 32 kg (70.5 lb) /cow/d), fat (1.24 kg (2.73 lb)/cow/d), and true protein (1.0 kg (2.2 lb)/cow/d) yields were detected between HDN and LDN farms, but the percentage of milk fat (3.98 vs 3.78%) and true protein (3.19 vs 3.08%) content were both higher on HDN farms. HDN farms had higher *de novo* FA, a trend for higher mixed origin, and no difference in preformed milk FA output/cow/day. This positive relationship between *de novo* FA and milk fat and true protein percentage agrees with previous results of Barbano et al. (2014) on bulk tank milk composition from 400 commercial dairy farms. The average fat and protein price for Federal Milk Order No. 1 for February through April 2015 (US Department of Agriculture, 2015) was \$4.19 and \$5.74 per kg (\$1.90 and \$2.61 per lb), respectively.

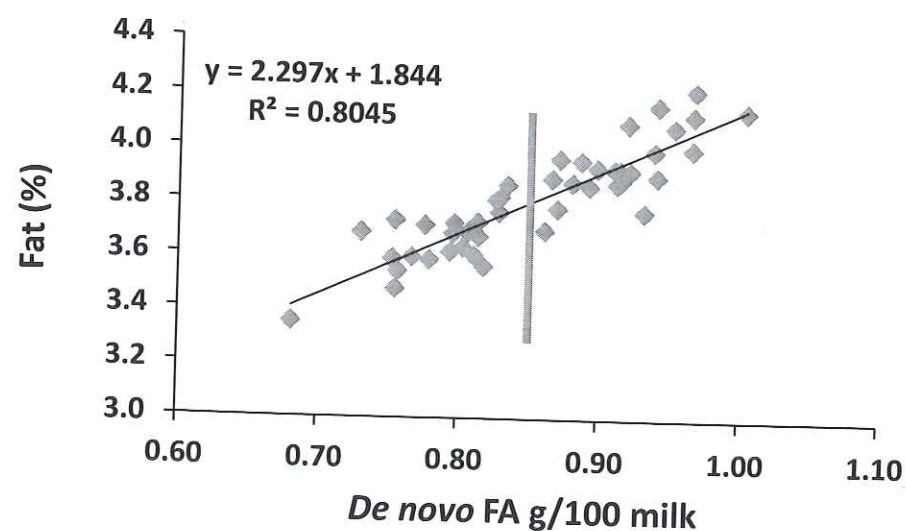
<sup>1</sup> Contact at: Department of Food Science, Cornell University, Ithaca, NY; E-mail: [dmb37@cornell.edu](mailto:dmb37@cornell.edu).



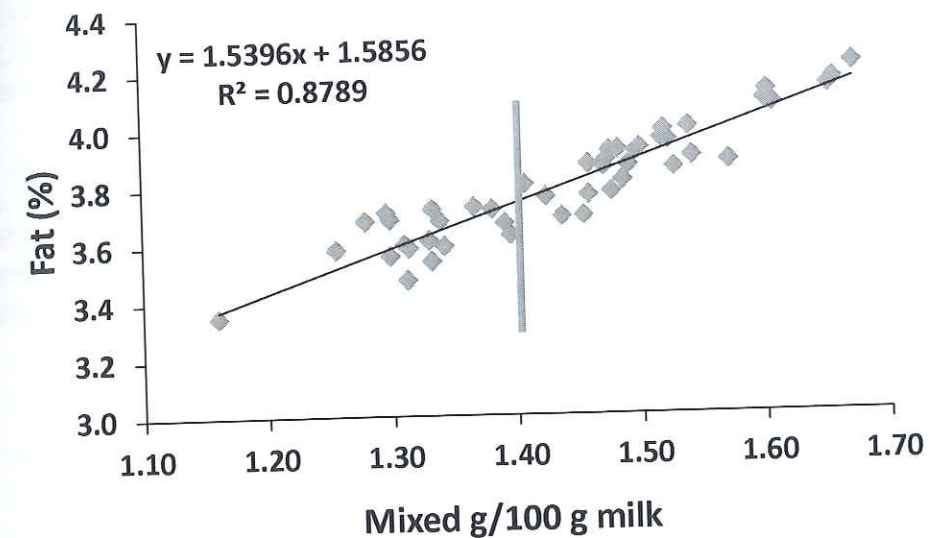
Therefore, at 66.1 lb (30 kg) of milk per cow per day, the average HDN farm earned a gross of \$5.00 and \$5.49 per cow for fat and protein, respectively. The average LDN farm at 30 kg of milk per cow per day earned a gross of \$4.75 and \$5.30 per cow for fat and protein, respectively. These differences for fat and true protein between HDN and LDN herds at 66.1 lb (30 kg) of milk would result in gross income differences of \$9,125 for fat and \$6,935 for true protein per 100 milking cows per year. Management (i.e., frequent feed delivery and increased feed bunk space per cow) and dietary (i.e., adequate physically effective fiber and lower ether extract) factors that differed between these HDN and LDN farms have been shown in earlier studies to affect ruminal function.

Based on data from these studies the following graphs (Figures 1 to 4) for Holstein farms were developed to help farms understand the relationships between bulk tank milk FA composition and bulk tank fat and protein tests.

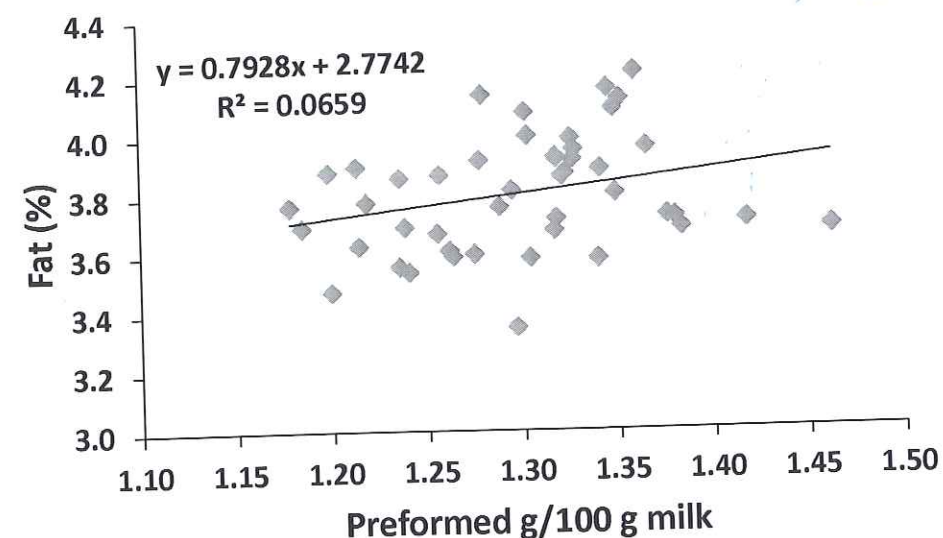
The data in Figures 1 to 4 indicate the relationship between milk FA composition and bulk tank milk fat test for Holstein herds. The verticle lines on the graphs indicate the relationship at a 3.75% fat test as bench mark. However, the data show clearly that Holsteins dairy herds are able to produce milk with much higher fat concentration, without sacrificing volume of milk production per cow (Woolpert et al., 2016, 2017). We are currently conducting a similar study with a group of Jersey farms and hope to graphs like this in Figures 1 to 4 for Jersey herds before the end of 2019. When enegy balance decreases, the relative proportion of *de novo* FA in milk fat will decrease and the relative proportion of preformed milk FA will increase. In bulk tank milk, you will see this change within 48 hours if there has been a error in ration formlation for a new ration or if something is restricting feed availability or feed intake by the cows.



**Figure 1.** Relationship of bulk tank milk fat test to concentration (g/100 g milk) of *de novo* FA in milk. In general, a farm needs to have a concentration of *de novo* FA higher than 0.85 g/100 g milk to achieve a bulk tank fat test higher than 3.75%.

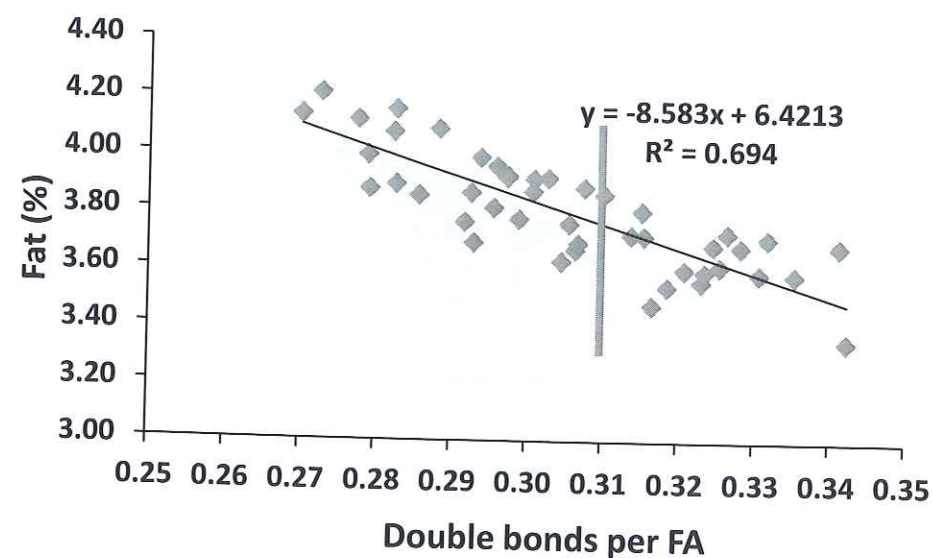


**Figure 2.** Relationship of bulk tank milk fat test to concentration (g/100 g milk) of mixed origin FA in milk. In general, a farm needs to have a concentration of mixed origin FA higher than 1.40 g/100 g milk to achieve a bulk tank fat test higher than 3.75%.



**Figure 3.** Relationship of bulk tank milk fat test to concentration (g/100 g milk) of preformed FA in milk. In general, the variation in preformed FA concentration in Holstein herds is less than *de novo* and mixed origin FA and is not well correlated with bulk tank milk fat test.





**Figure 4.** Relationship of bulk tank milk fat FA unsaturation with bulk tank milk fat test. As double bonds per FA increases the bulk tank milk fat test decreases. To achieve a 3.75 % fat test a farm needs to have a double bond per FA of less than 0.31.

Starting in February of 2016, information on FA composition of bulk tank milk was provided to the individual producers of the St Albans Cooperative (Vermont) along with their payment test data on the same milk samples and in the summer of 2017 Agrimark Cooperative (Springfield, MA) and Cayuga Milk Ingredients (Auburn, NY) have started providing similar data to their producers on the official bulk tank milk samples that are used for milk payment testing.

In addition, in the last 2 years we have expanded our milk analysis research on FA analysis to individual cow milk samples at Cornell and in collaboration with Miner Institute in Chazy, NY. This paper will focus on the use of milk FA information for feeding management of dairy cows at the bulk tank level and report the status of our work on individual cow data with respect to how these milk composition and production parameters change with stage of lactation for primiparous and multiparous cows.

### Experimental Approach

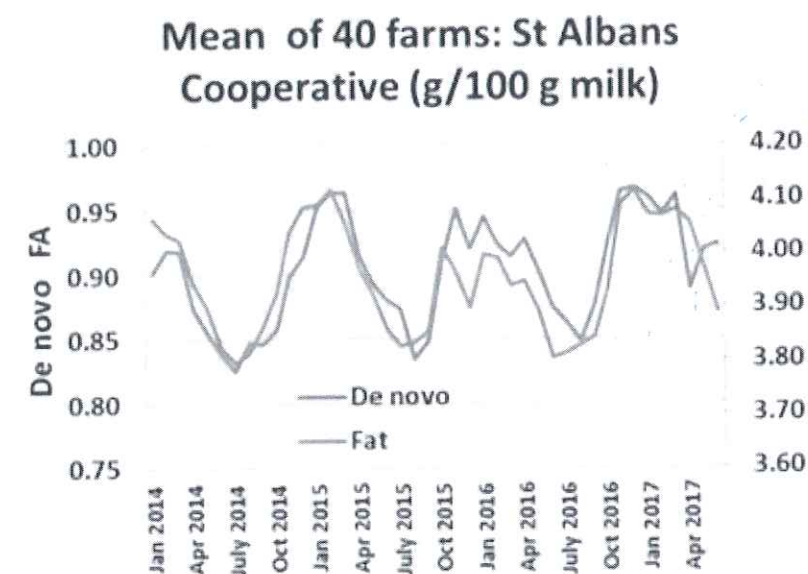
Partial least squares (PLS) chemometric prediction models for FA were developed from MIR spectra in the Cornell University laboratory using a Delta Instruments Lactoscope (Delta Instruments, Drachten, Netherlands) and have been described in detail by Wojciechowski and Barbano (2016). Data collection has continued at the St Albans Cooperative and within farm seasonality patterns of bulk tank milk fat, protein, and milk FA composition has been measured using the routine milk FA analysis by MIR. In addition, in the past year, bulk tank milk sampling has been done on a wide range of farms from various regions of the US to confirm if the same milk fat, protein and milk FA composition relationships are observed in bulk tank milks from different regions of the US. These samples were collected daily for 5 to 7 days on each farm, preserved and

refrigerated. At the end of the collection period, the milk samples were shipped on ice to Cornell University for MIR analysis and spot checking FA composition with GLC analysis, particularly to obtain more detail about milk trans FA levels at each farm.

For individual cow milk analysis we are conducting an intensive study at Miner Institute. We have a high speed MIR milk analysis system on site testing milk from individual cows. The routine fresh milk testing is done one day per week, 3 milkings in a row on each cow in the herd. The goal is to build stage of lactation curves for all the new milk analysis parameters on both a concentration basis and a daily output per cow basis.

### Results

**Seasonality of Bulk Tank Milk.** Over the past 3 to 4 years we have followed the pattern of seasonality of milk fat and protein in relation to milk FA composition on a group of 40 farms with the St. Albans Cooperative (Figures 5 to 8). The data are from the routine testing results using MIR in the St. Albans Cooperative on fresh bulk tank milk samples used for payment testing.



**Figure 5.** Seasonality of milk fat and *de novo* FA in milk.



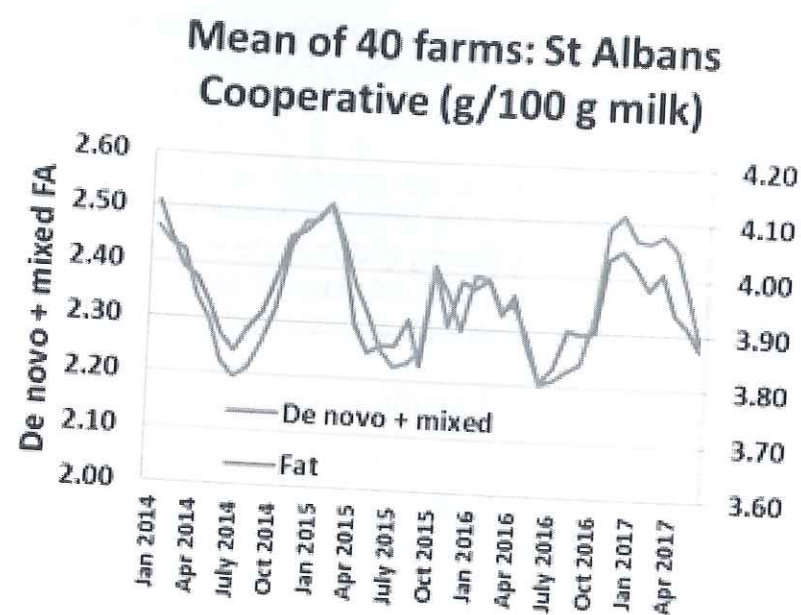


Figure 6. Seasonality of milk fat and *de novo* + mixed origin FA in milk

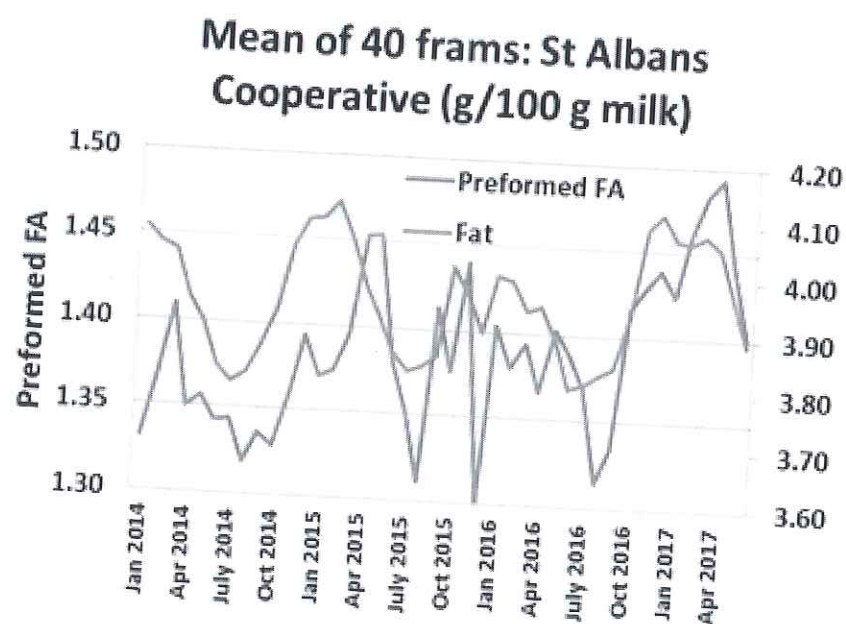


Figure 7. Seasonality of milk fat and preformed FA in milk.

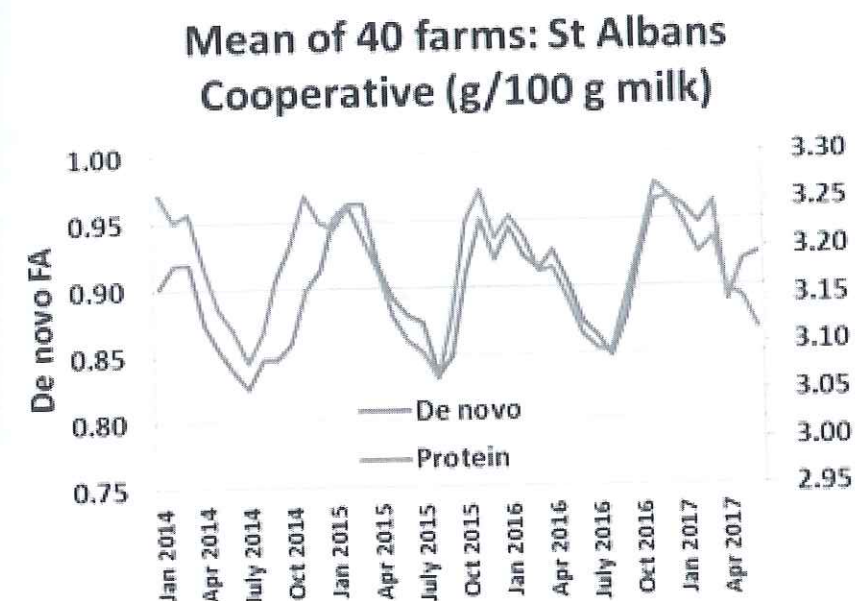


Figure 8. Seasonality of milk protein and *de novo* FA concentration in milk.

The seasonality of *de novo* and mixed origin milk FA concentration follows the seasonal pattern of milk fat and protein variation while variation in preformed fatty FA in milk does not. Much of the variation in the mixed origin FA concentration is probably due to variation in the portion of the mixed origin FA produced by *de novo* synthesis from acetate and butyrate from forage digestion in the rumen. These seasonal changes may be related to time and temperature induced changes in the fermentation of corn silage, starch degradability, forage quality and heat stress.

**Herd to herd variation in milk composition in North America.** Over the past year bulk tank milk samples were collected from large and small Holstein farms from different regions of the US. Each bulk tank or tanker within the farm was sampled each day for 5 to 7 day periods and milk samples were sent to the Cornell University laboratory for MIR and GLC analyses. There were some grazing herds, organic herds, and very large conventional herds in the population with a wide range of milk production per cow and milk composition. The relationship between bulk tank milk composition and FA composition is shown in Figures 9 to 13 for 167 farms.

The relationship between *de novo* and *de novo* plus mixed origin observed in bulk tanks milk produced by farms from across the US are similar those found for Holstein herds in the Northeast. A level of about 0.85 g *de novo* FA per 100 g of milk will achieve about a 3.75% fat test (as seen by comparison of Figure 1 versus Figure 9). The same general relationship is seen in both data sets. Another data set of 500 farms from the Texas/New Mexico area shows similar patterns (data not shown). Milk fat and protein output per cow per day are also strongly associated with total weight of milk produced per day. Those relationships are shown in Figures 11 and 12.



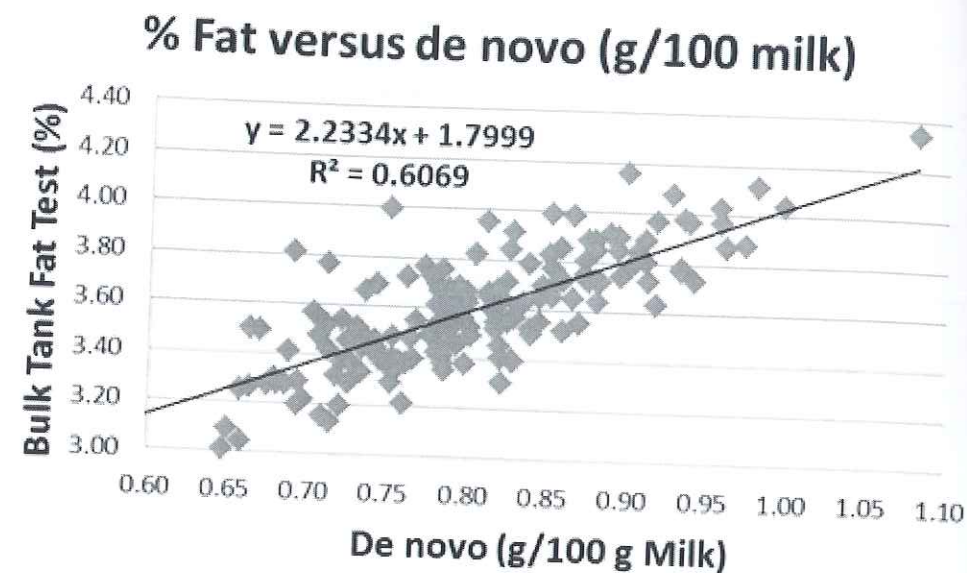


Figure 9. Relationship between bulk tank fat and *de novo* FA concentration (167 farms).

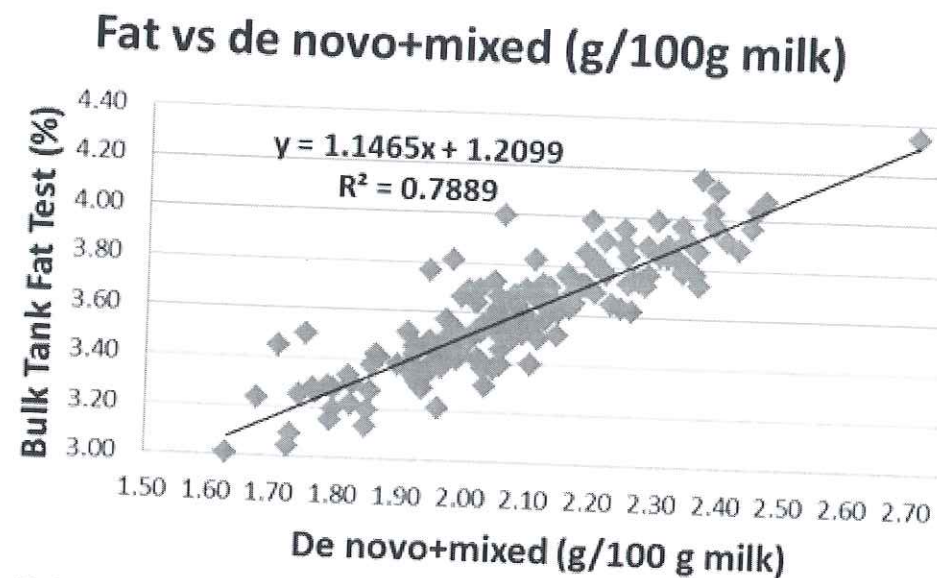


Figure 10. Relationship between bulk tank fat and *de novo* + mixed origin FA (167 farms).

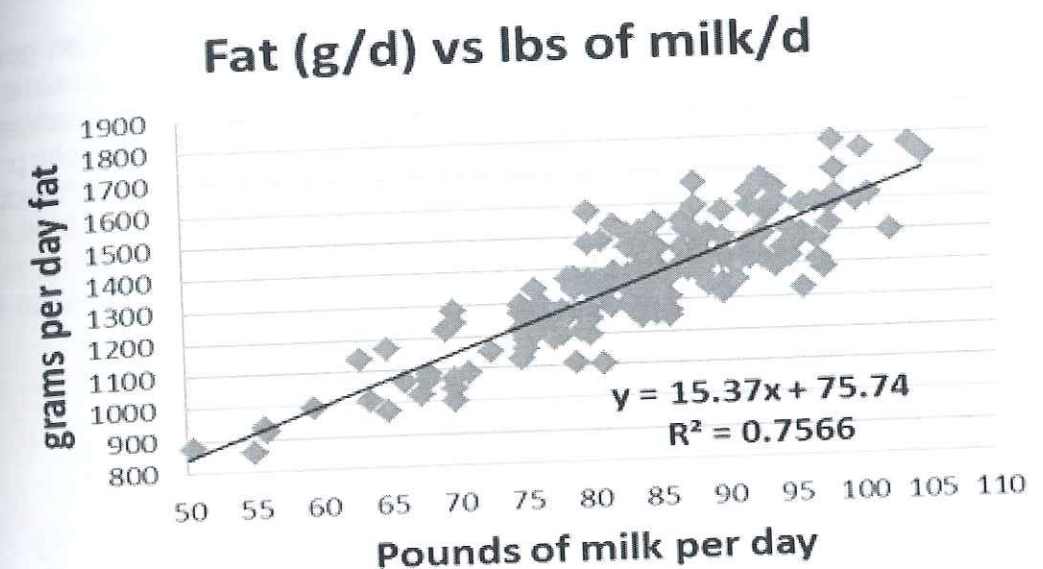


Figure 11. Grams of fat per cow per day and milk production (167 farms).

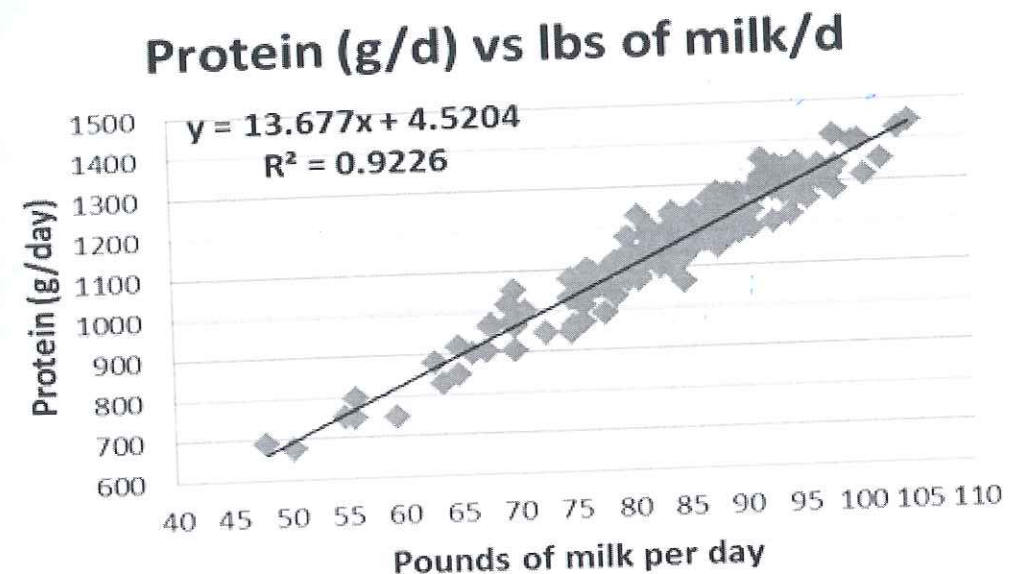


Figure 12. Grams of protein per cow per day and milk production (167 farms).

Overall, dairy cows have the potential to produce more grams of fat and protein per day if they produce more milk. But what drives milk production? The synthesis of lactose and increasing the grams per day output of lactose is fundamental to producing more pounds of milk per day. How often do we think about or look at how much lactose is being produced per cow per day? Does my lab even report a value for lactose and is the lactose value correct? Because there is no payment based on lactose nutritionists may ignore it. Lactose production (grams per cow per day) is highly dependent on glucose metabolism in the cow. To produce more milk per cow, more lactose per day needs to be produced, as shown in Figure 13. The correlation is very strong. If you want to achieve 90 to 100 lb (40.9 to 45.4 kg) of milk, the cows need to be producing between



1900 and 2100 grams of lactose per day. Generally, when milk production goes down and grams of lactose synthesized per day goes down, it is an indication that either energy intake has gone down or some other health related factor (e.g., mastitis, ketosis, leaky gut, etc.) has caused an immune system response that has a higher metabolic priority for use of glucose and as a result of this lactose synthesis and milk volume decreases.

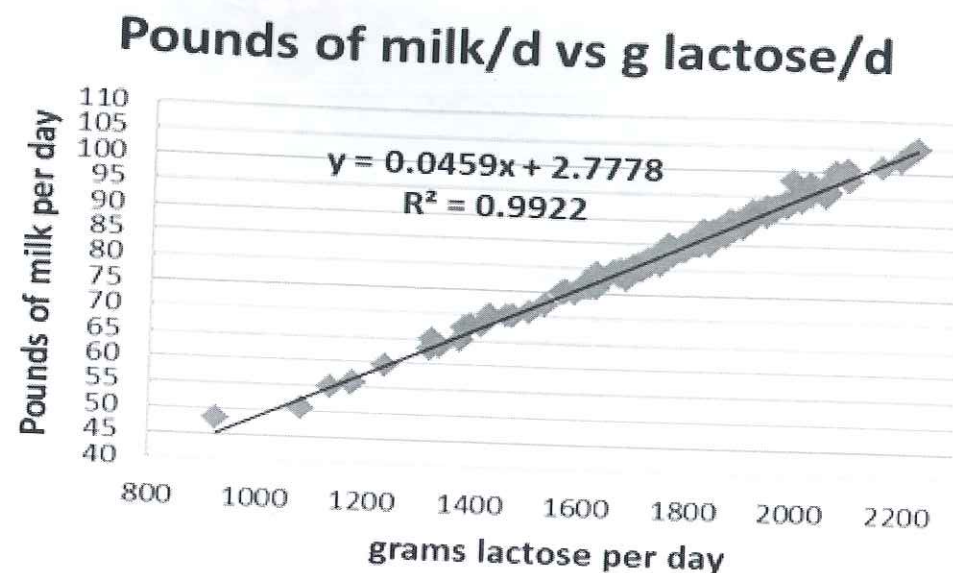


Figure 13. Grams of lactose produced per cow per day and milk production (167 farms).

As this new milk testing technology becomes more widely available in the dairy industry it is likely to be used as a herd management tool to test milk from different feeding groups of cows that may have a very different number of days of milk (DIM) from one group to another or have a different parity status from one group to another. Both DIM and parity influence milk and milk FA composition. There are large changes in milk FA composition with stage of lactation, particularly during the transition period. When looking at milk composition and FA composition, differences in parity or stage of lactation needs to be taken into account when interpreting data. As a result, we have been collecting data at the Miner Institute to produce lactation curves on all of these milk parameters.

**Stage of Lactation.** The concentrations of FA in milk changes with DIM and the changes are particularly large in early lactation when the cow is in negative energy balance. During this period it is normal for the preformed FA to be high and the mixed and *de novo* FA to be low. However as dry matter intake increases after calving, the milk FA composition should change quickly if the cow's blood NEFA concentration decreases normally. If milk sampling and testing for FA is being done on different groups of cows within a herd, then these stage of lactation changes need to be considered to properly interpret that data. The graphs below (Figures 14 to 17) are stage of lactation data collected from cows over a period of 3 years at the Miner Institute. The Miner Institute Holstein herd milked is 3 times per day. In July 2017 the DHI test results were: RHA of 29,711 lb (13,489 kg) milk, 1261 lb (572 kg) fat, 908 lb (412 kg) protein, 104,000 cells/mL weighted SCC, 94.6 lb (42.95 kg) test day milk/cow, 167 DIM, and 376 cows milking (388 yearly rolling average). Lactating diets are typically 50 to 60% forage with at least 2/3 of

forage coming from corn silage. Grain mixes typically contain corn grain, soybean meal, commercial soy/canola products, byproducts, rumen inert fat, plus mineral and vitamin supplements. Diets are balanced for lysine and methionine.

The change in g/100 g milk of *de novo*, mixed, and preformed FA with week of lactation is shown in Figure 14 and the relative percentages are shown in Figure 15 for the Miner Institute herd producing an average of about 92 lb (41.8 kg) per cow per day on TMR feeding system.

### De novo, mixed and preformed FA

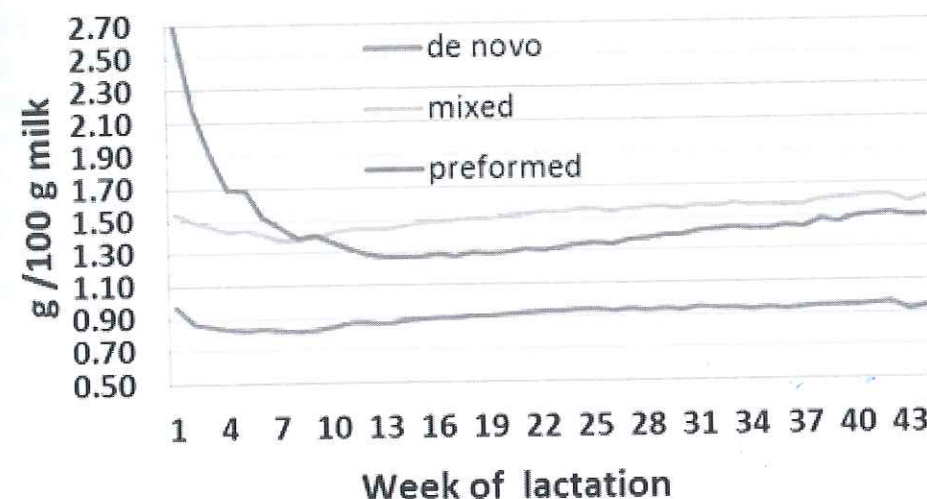


Figure 14. *De novo*, mixed, and preformed FA (g/100 g milk) over lactation for all cows.

### De novo, mixed, preformed FA

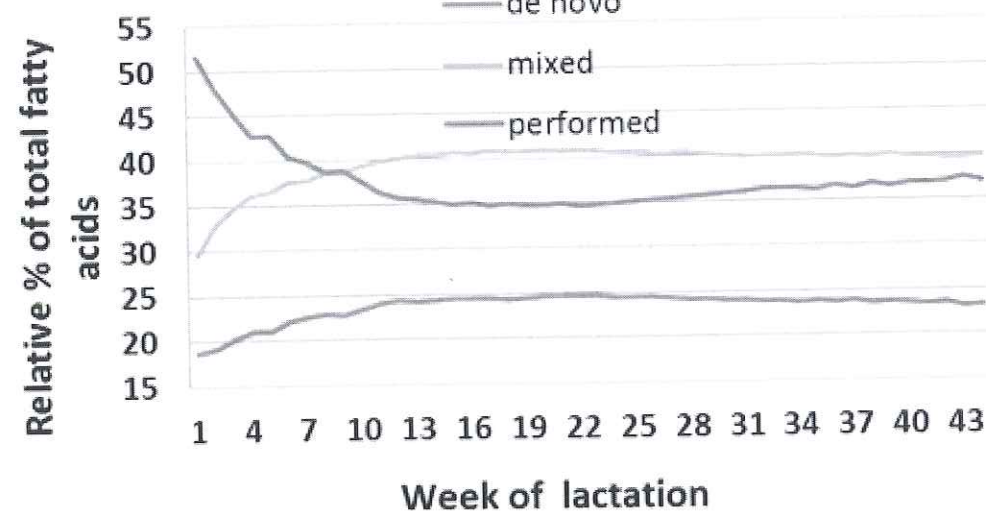


Figure 15. *De novo*, mixed, and preformed FA (relative %) over lactation for all cows.



There are large changes in milk FA composition during the first 10 weeks of lactation on both a g/100 g milk and relative percentage basis with the preformed FA being high at the beginning of lactation and decreasing to relatively stable levels by about 10 weeks of lactation. When testing milk on larger farms from groups of cows that differ in stage of lactation, these changes in milk FA composition with stage of lactation need to be considered when interpreting data along with information on milk production per cow per day, cow health, milk SCC, feed composition, and dry matter intake.

Interpretation of results from a management point of view becomes even more interesting when the data are converted to grams per day per cow output. The weight of FA divided by 0.945 is approximately equal to the fat test (g/100 g milk). This factor assumes that milk fat is about 5.5% by weight glycerol and 94.5% by weight FA. Figure 16 represents the average of all cows in the herd, but the stage of lactation graph for grams per cow per day is very different for first parity versus older cows. When evaluating performance of older versus younger cows, this factor needs to be considered. The difference between multi and primiparous cows for output of *de novo* and preformed FA per cow per day is shown in Figure 17. The output of all groups of FA in grams per cow per day is much more stable over time for primiparous cows versus older cows. The older cows have much higher preformed FA output per cow per day in early lactation due to body fat mobilization.

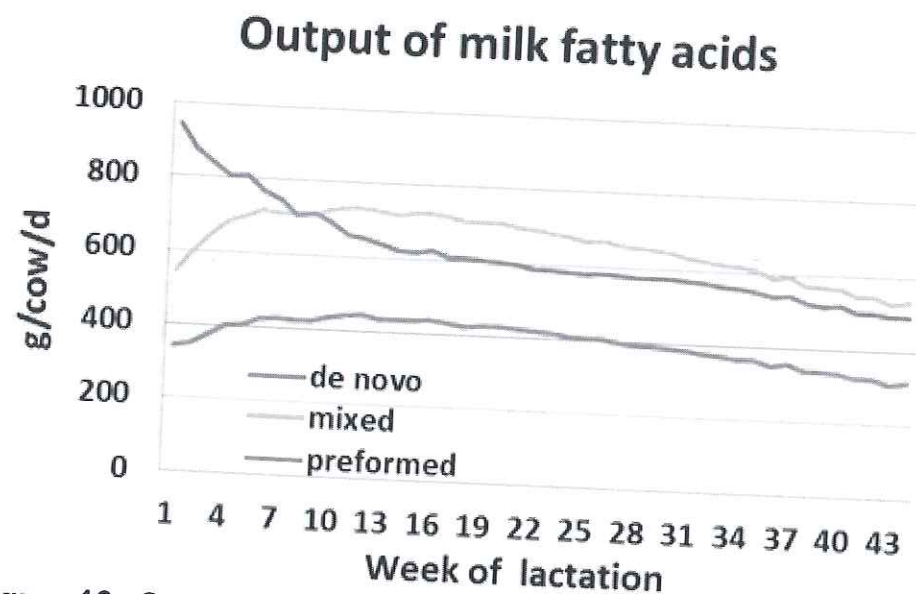


Figure 16. Stage of lactation production graph for all cows (g/cow/day).

## Multi & Primi Parous Cow Fatty Acids

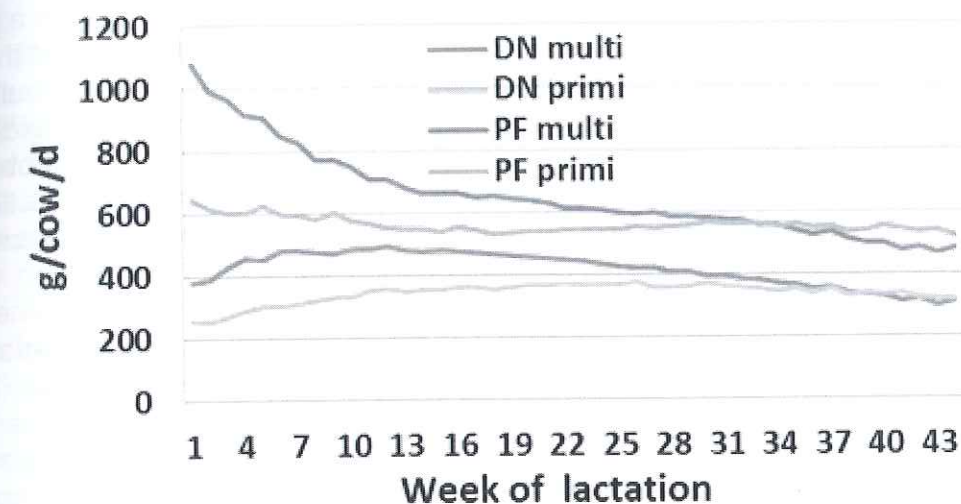


Figure 17. Stage of lactation: *de novo* and preformed FA for primi and multiparous cows.

## Interpretation of Field Data on Bulk Tank Milks: Whole Herd Diagnostic

Milk FA data will become more commonly available on bulk tank milks as milk payment testing laboratories adopt this new milk testing technology in combination with existing metrics of milk composition and milk quality. Given the factors shown above and the wide range of differences in farm management conditions and feeding, the data need to be interpreted with caution and complete knowledge of the management and ration on each farm is essential. Given those cautions, the new milk analysis data add a powerful new opportunity in precision management of milk production.

In looking at the bulk tank data from the 167 farms (Figures 9 to 13), the following questions and relationships in the data start to become apparent. For milk composition from an individual farm the following data are useful for the full herd or for groups of cows:

- Milk per cow per day
- Milking frequency (2X or 3X) – milk and component output expected to be 10 to 15% higher on 3X farms
- Milk SCC (cells/mL)
- Milk MUN (mg/dL or mg/100 g milk)
- Milk fat unsaturation (double bonds per FA)
- Milk fat (g/100 g milk and g/day production)
- Milk protein (g/100 g milk and g/day production)
- Milk lactose (g/100 g milk and g/day production)
- Milk *de novo* FA (g/100 g milk and g/day production)
- Milk mixed origin FA (g/100 g milk and g/day production)
- Milk preformed FA (g/100 g milk and g/day production)



An example of how to look at the data and questions to ask:

**Milk somatic cell count: cells/mL.** What is the bulk tank milk SCC over a period of time? The bulk tank should be <200,000 cell/mL. If > 300,000 cell/mL, look at the milk lactose in g/100 g milk. If the lactose is 4.65 g/100 g milk or higher, the high bulk tank SCC is likely to be caused by a very small number of individual cows in the herd/group with very high SCC, while if the lactose is low (< 4.60 g/100 g milk) there is probably a more wide spread (i.e., more cows) incidence of cows with intramammary infections. If the herd has a wide spread mastitis problem, that problem needs to be addressed first because it is negatively impacting the production of the herd.

**Milk urea nitrogen: mg/dL.** What is the concentration and day to day variation in MUN? If the MUN is >14 to 16, it is likely that rumen ammonia levels are too high. Lower ration input of dietary degradable protein or increasing available carbohydrates in the ration should be considered depending on the context of the complete ration composition. Another aspect of MUN is to look at the day-to-day variation in MUN within the same farm. MUN decreases rapidly when cows do not have access to feed. Thus, day to day variation in MUN within the same farm is an index of how consistently the farm is keeping feed accessible to cows on a continuous basis (i.e., feed bunk management).

**Milk fat unsaturation: double bonds per FA.** This is a useful index of what is happening in the rumen, but is less of a driver and more of a correlated outcome of other things that are happening. In general, as double bonds per FA increases, milk fat decreases (Figure 4). A rule of thumb based on our observations for Holsteins is that when the double bonds per FA is > 0.31, the probability of *trans* FA induced milk fat depression is greatly increased. A word of caution is that there is a large stage of lactation impact on double bonds per FA and cows in the transition period will have a high double bond per FA without having *trans* FA induced milk fat depression. Thus, be careful with interpretation of milk fat unsaturation on groups of early lactation cows.

**Lactose: grams per cow per day.** Making more lactose per day (anhydrous lactose, not lactose by difference) makes more milk per day (see Figure 13). To have a high output of lactose per cow per day, glucose supply, transport, and metabolism needs to be working very well. Without increasing lactose production in a Holstein cow, you cannot increase milk. Thus, figuring out how to manage cows to produce lactose is the key to getting more milk per cow per day and is partially correlated higher outputs of fat and protein per cow per day. Factors to consider are the production of propionate produced in the rumen and the undegraded starch that is leaving the rumen and available in the lower gastrointestinal tract. Also, is there some cow health issue (immune system activation) or environmental factor (e.g., heat stress) in the herd that is putting a demand on the glucose supply and reducing the glucose available for milk synthesis?

When daily milk yield per cow is low in a Holstein herd, is synthesis of lactose the first thing a dairy nutritionist thinks about? It should be. If a 3X Holstein multiparous cow is going to produce a lactation average of > 85 lb (38.6 kg) of milk per day, she is going

to need to produce at least an average of 1800 grams of lactose per day. This is the foundation upon which to build high fat and protein output per cow per day.

**De novo and mixed origin FA: g/100 g milk.** There is a strong correlation between changes on *de novo* FA concentration in milk and bulk tank milk fat and protein tests (Figures 1, 2, 5, 6, 9 and 10). It is thought that the basis for the correlation between *de novo* FA and milk protein (Figure 8) is due to the higher microbial biomass that provides essential amino acids in support of milk protein synthesis in combination with rumen undegradable protein. For multiparous cows, stage of lactation has a large impact on *de novo* and mixed origin milk FA production. By pass feeding of palm-based fat supplements may also increase the mixed origin FA content of in milk (Piantoni et al., 2013). In general, when *de novo* (> 0.85 g/100 g milk) and mixed origin FA (>1.35 g/100 g milk) are high, it is an indication that rumen fermentation of carbohydrate is working well and the supply of volatile FA from the rumen is good. This can be the case with either a high or lower level of milk (i.e., lactose) production. Fixing the low lactose production issue will likely allow the cows to maintain high concentration of *de novo* and mixed origin but increase per day output of fat and protein given an adequate supply of their precursors.

**Preformed FA: g/100 g milk.** The preformed FA do not normally vary so much within a herd across time in the bulk tank (1.2 to 1.4 g/100 g milk), unless there is some major change in diet/nutrition. However, it does change dramatically with stage of lactation and it can be very high for multiparous early lactation cows (Figures 14 and 15). As we have more experience with the milk FA metrics in the field, it may lead to strategies of using a different chain length of by-pass fat at different stage of lactation to better support maintenance of body condition and milk production at the appropriate times during lactation.

**Fat and protein percent and g/cow per day.** For multiparous cows, stage of lactation has a large impact on both parameters. Generally fat and protein in g/ 100 g milk and grams output per cow per day will be higher when *de novo* and mixed origin FA are high. Focusing on feeding and nutrition factors that support high production per cow per day of *de novo* and mixed origin FA and lactose will maximize both milk fat and protein output per cow per day if there is an adequate supply of essential amino acids to support milk protein synthesis.

## Conclusions

Data from routine high frequency (i.e., daily) bulk tank milk component, SCC, and milk FA testing combined with milk weight per cow for whole herd diagnostic analysis of overall nutritional and management status of dairy herds. The testing was done using MIR as part of the routine milk payment testing. The advantage of this approach is that no additional sampling collection cost is required, the instrument that does the milk FA analysis can be the same instrument that produces the milk fat and protein test result, and it does not take any longer to test each milk sample. There would be additional cost to purchase reference milk samples for calibration of the FA parameters for the MIR milk analyzer. The positive correlation between increased *de novo* FA synthesis and bulk tank



milk fat and protein concentration can be used as an indicator of the quality and balance and the rumen fermentation of carbohydrates and if changes in feeding and management are impacting *de novo* synthesis of milk fat. Seasonal variation in whole herd milk fat and protein concentration was highly correlated with seasonal variation in *de novo* FA synthesis. Milk FA composition changes with both DIM and differs between primi and multiparous cows. Milk FA testing and this diagnostic approach could be applied to testing milk from large feeding groups of cows within the same farm, if representative feeding group milk samples can be collected and tested and the milk produced per cow is known. For feeding group or individual cow milk testing care must be taken to consider the milk weight per cow per day, diet composition, dry matter intake, DIM and parity into the interpretation of the milk composition data.

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## SESSION NOTES



# Lipid Mobilization and Inflammation During the Transition Period

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## Introduction

Lipid mobilization is a metabolic process that includes lipogenesis and lipolysis. Within the adipose tissue (AT), lipogenesis is the assembly of triglycerides through a stepwise addition of fatty acids catalyzed by glycerol-3-phosphate acyltransferase, 1-acylglycerol-3-phosphate acyltransferase, lipins, and diacylglycerol acyltransferase (Takeuchi and Reue, 2009). During lipolysis, adipocyte's adipose triglyceride lipase, hormone-sensitive lipase, and monoacylglycerol lipase hydrolysis the triglyceride molecule into glycerol and nonesterified fatty acids (NEFA) [reviewed by Arner and Langin (2014)]. Released NEFA are either re-esterified to triglycerides through lipogenesis or exported into circulation where they are transported by albumin and Fetuin-A for use in other tissues as fuel or secreted in milkfat (Strieder-Barboza et al., 2018). During the transition period, the net release of NEFA from AT into circulation is the result of reduced lipogenesis and enhanced lipolysis within adipocytes (De Koster et al., 2018). Normally, lipolysis decreases and lipogenesis replenishes adipocytes' triglyceride stores as lactation progresses. However, when AT exhibits an impaired response to the anti-lipolytic effects of insulin, lipolysis becomes intense and protracted, and lipogenesis is shut down. Excessive lipolysis increases the risk for inflammatory and metabolic diseases and reduces lactation and reproductive performance. Among the mechanisms driving these deleterious effects, there are alterations in the inflammatory responses that lead to dysregulation of metabolic and immune functions within the AT and systemically.

## Periparturient Adipose Tissue Remodeling, An Inflammatory Process Induced by Lipolysis

The consequences of enhanced lipolysis and reduced lipogenesis in AT of transition cows go beyond the release of NEFA into circulation. Excessive lipolysis also induces a remodeling process in the adipose organ that is characterized by macrophage infiltration and changes in its extracellular matrix (Contreras et al., 2017b).

Macrophages are the most abundant immune cell type in the AT of ruminants and comprise 5-10% of its stromal vascular cell (i.e., non-adipocytes) fraction (Ampem et al., 2016). In dairy cows, lipolysis enhances macrophage trafficking into AT during the transition period and in feed restriction-induced negative energy balance (Contreras et al., 2016, Vailati-Riboni et al., 2017, Newman et al., 2018). In cases where lipolysis is severe, such

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as in displaced abomasum and ketosis, macrophages account for 20% of cells in the stromal vascular fraction or 2% of the total number of cells in AT (Contreras et al., 2015, Häussler et al., 2017). The role of AT macrophages during lipolysis is to contain and eliminate the highly cytotoxic lipolytic products that include NEFA, diglycerides, and monoglycerides (Lee et al., 2013).

Adipose tissue macrophages are broadly classified as classical (M1), which have active pro-inflammatory responses, and alternative (M2), which promote inflammation resolution. At any given time, macrophages within the AT are a mixture of M1, M2, and intermediate phenotypes. In periparturient cows with excessive lipolysis, including those with displaced abomasum and ketosis, AT macrophages are predominantly of the M1 phenotype and accumulate in aggregates within omental and subcutaneous depots (Contreras et al., 2015, Newman et al., 2018). In contrast, during moderate lipolysis induced by a short four-day feed restriction protocol in late-lactation cows, macrophage infiltration into the same AT depots occurs, but without any change in phenotype (Contreras et al., 2016).

The total mass of the AT of dairy cows is drastically reduced during the transition period. As the size of the adipose organ is diminished, its extracellular matrix composition is altered. Transcriptomics studies indicate that ruminants with higher lipolysis rate immediately after parturition have a stronger expression of collagen type I and III in AT compared to those with low lipolysis intensity (Faulconnier et al., 2011, Akbar et al., 2014). Expression of thrombospondin 1, an important extracellular matrix protein, is also upregulated during the first two weeks after calving when lipolysis rate reaches its peak [reanalysis of microarray data from Sumner-Thomson et al. (2011)]. Higher content of collagen type III and thrombospondin 1 in AT has been associated with impaired sensitivity to insulin by adipocytes (Buechler et al., 2015, Matsuo et al., 2015).

In transition cows, the inflammatory responses driven by AT remodeling perpetuate the lipolytic stimuli. Adipose tissue macrophages and other immune cells that are active during AT remodeling express and secrete potent blockers of insulin signaling including interleukin (IL) 1- $\beta$ , IL-6, resistin, and tumor necrosis factor (TNF) - $\alpha$  (Martinez-Santibanez and Lumeng, 2014). This AT-specific insulin resistance was demonstrated by Zachut and colleagues in a group of transition dairy cows (Zachut et al., 2013). In their study, cows that lost more body condition during early lactation exhibited a significant reduction in the phosphorylation of downstream insulin signaling pathways, such as IRS-1 and AKT, compared to cows with low lipolysis and reduced weight loss. Remarkably, the activation of these insulin signaling pathways remained intact in the liver.

## Lipolysis Products as Modulators of Inflammation

Fatty acids released during lipolysis can modulate the inflammatory phenotype of macrophages and other immune cells. For example, saturated FA induce an M1 like inflammatory phenotype in macrophages of the AT and other organs through the activation of Toll-Like Receptors (TLR) 1, 2, 4, and 6 (Velloso et al., 2015). Saturated FA such as lauric, myristic, and palmitic strongly activate TLR4 and enhance the secretion of monocyte



chemotactic protein-1 (**MCP-1**) (Han et al., 2010). Importantly, these saturated FA are preferentially mobilized from AT during lipolysis (Douglas et al., 2007, Contreras et al., 2017a).

Polyunsaturated fatty acids released during lipolysis modulate immune function and inflammation through their oxidation products (oxylipids). For example, linoleic acid is oxidized by 15-lipoxygenase (**LOX**) and by other non-enzymatic reactions to produce hydroxyl-octadecadienoic acids (**HODEs**). The molecule 13-HODE, a product of lipoxygenases and cyclooxygenases, promotes M2 polarization during lipolysis and acts as a PPAR gamma ligand that promotes adipogenesis and lipogenesis (Lee et al., 2016). In contrast, 9-HODE, a product of non-enzymatic oxidation by reactive oxygen species, promotes M1 polarization in tissues with lipid infiltration like vessels with atherosclerotic vessels (Vangaveti et al., 2010).

In dairy cows, linoleic acid is the most abundant polyunsaturated fatty acid in plasma and in AT, and it is preferentially mobilized by lipolysis during the transition period (Contreras et al., 2010). The dynamics of the plasma and AT content of its derived oxylipids are linked with lipolysis intensity. In healthy transition cows, plasma 13-HODE increases at 1 week after parturition from its levels at 1 week before calving. In contrast, 9-HODE, an indicator of oxidative stress, remains unchanged. In AT, 9-HODE tends to increase after parturition and 13-HODE is higher than at either 1 or 4 weeks before calving. Adipose tissue content of 13-HODE is positively associated with plasma beta-hydroxybutyrate concentrations (Contreras et al., 2017a). In the future, HODEs and oxylipids derived from other polyunsaturated fatty acids could be used as disease risk or lactation performance predictors in transition dairy cows. However, the dynamics of the synthesis of lipolysis products in transition dairy cows are poorly understood and should be the focus of future research.

### Lipolysis and Immune Function

Excessive lipolysis impairs the efficacy of the inflammatory responses of cells from both the innate and adaptive immune systems [reviewed by Contreras et al. (2018)]. For example, cows challenged with *Strep. uberis* (intramammary) and with high lipolysis rates induced by feed restriction, exhibit an increased number of immature polymorphonuclear cells in circulation that have lower phagocytic activity compared with cows in positive energy balance (Moyes et al., 2009). In transition cows, high lipolysis rates are associated with reduced chemotactic activity and impaired phagocytosis in neutrophils (Nonnecke et al., 2003, Hammon et al., 2006). The same population of cells has limited oxidative burst when circulating NEFA are above 500  $\mu$ M and its viability is drastically reduced when NEFA concentrations reach >1.0 mM (Scalia et al., 2006; Ster et al., 2012).

The inflammatory response of macrophages and lymphocytes are also affected by excessive lipolysis during the transition period. Exposure to high NEFA concentrations reduces the mitogenic capacity of these mononuclear cells and limits their secretion of IFN- $\gamma$  and IgM (Lacetera et al., 2005, Ster et al., 2012). High NEFA affect the function of cells of the adaptive immune system. High lipolysis increases B lymphocytes

populations and reduces  $\gamma\delta$  T lymphocytes. Reduced  $\gamma\delta$  T lymphocytes are associated with deficient immune responses in epithelial tissues (Pollock and Welsh, 2002). Collectively, these studies indicate that excessive lipolysis augments disease susceptibility in transition dairy cows by impairing the inflammatory responses of innate and adaptive immune cells and reducing their capacity to clear pathogens.

### Adipokines Modulate Immunity and Metabolism

Besides NEFA and other lipolysis products, AT also modulates inflammatory processes and the immune and metabolic function of transition dairy cattle through the secretion of adipocyte-derived peptides (i.e., adipokines). Although there are over 100 adipokines described in rodents and humans, only adiponectin, leptin, and resistin are well characterized in transition dairy cows.

Adiponectin enhances insulin sensitivity in adipocytes, hepatocytes, and muscle cells. At the same time, this adipokine promotes fatty acid  $\beta$ -oxidation in the liver and the skeletal muscle. In transition dairy cows, plasma adiponectin concentrations are reduced during the first week after parturition compared to levels observed during the dry period and peak lactation (Kabara et al., 2014). In addition to metabolic effects, adiponectin modulates the inflammatory responses of human and bovine macrophages by reducing their expression and secretion of TNF alpha and other pro-inflammatory cytokines (Kabara et al., 2014). Adiponectin is also an important modulator of adaptive immunity as it is required for dendritic cell activation and T-cell polarization (Jung et al., 2012). Excessive AT inflammation reduces the secretion of adiponectin by adipocytes thus impairing the use of NEFA as an energy substrate in the liver and skeletal muscle.

Leptin modulates the inflammatory responses locally and systemically. Hypoleptinemia impairs the efficacy of T cell immune responses by promoting a shift in the phenotype of these cells from type 1 (pro-inflammatory) to a T2 helper. This phenotype change reduces the capacity for pathogen clearance. Leptin is also necessary for adequate maturation and inflammatory responses in dendritic cells. In macrophages and polymorphonuclear cells, leptin signaling is required for phagocytosis in response to toll-like-receptor activation (Naylor and Petri Jr, 2016). Similar to adiponectin, leptin reaches its nadir during the first week after calving, while the highest plasma concentrations are observed early during the dry period (Chilliard et al., 2005).

Resistin is another adipokine with the capacity to modulate immune and inflammatory responses systemically. In dairy cows, plasma resistin peaks during the first week after calving and returns to prepartum levels by five weeks in milk (Reverchon et al., 2014). In humans and rodents, resistin expression in adipocytes is stimulated by IL-6, hyperglycemia and growth hormone. Resistin impairs insulin signaling in adipocytes and is characterized as a pro-inflammatory adipokine (AL-Suhaimi and Shehzad, 2013). In bovines, resistin promotes lipolysis in adipocytes, but its effects on immune cells are currently unknown.



## Modulating Lipid Mobilization in the Transition Period

Reduced lipogenesis and increased lipolysis are homeorhetic adaptations to negative energy balance that maintain energy availability for milk production. Although the process of lipid mobilization is affected by physiological, nutritional, genetic, management factors, there are different on-farm management, nutritional, pharmacological tools that can be used to limit lipolysis and could potentially promote lipogenesis [reviewed in (Contreras et al., 2018)].

A basic management and nutritional strategy that reduces lipolysis and promotes lipogenesis in the transition period is maximizing dry matter intake (DMI). In addition, it is necessary to limit the sudden drop in feed intake commonly observed during the final weeks of the dry period (Grummer et al., 2004). It is also imperative to balance prepartum diets to meet but not exceed energy requirements. This is usually accomplished by feeding high levels of fiber (Allen and Piantoni, 2014). When balancing rations for dry cows, it is necessary to take into account that overfeeding energy in the last weeks of gestation enhances lipolysis postpartum and increases the risk of fatty liver (Douglas et al., 2006). Cows that gain excessive BCS during the dry period have larger adipocytes that are more sensitive to lipolytic stimuli postpartum (De Koster et al., 2016). An additional feeding strategy is to boost the production of ruminal propionate postpartum by feeding high amounts of moderately fermentable starch (van Knegsel et al., 2007). This nutritional intervention limits AT lipolysis by enhancing insulin secretion (McCarthy et al., 2015).

To complement ration balancing strategies, the inclusion of nutritional supplements that limit lipid mobilization in the diet of transition cows can be considered. Feeding niacin (as nicotinic acid) reduces AT lipolysis by limiting the phosphorylation of hormone-sensitive lipase (Kenez et al., 2014). However, niacin supplementation has shown inconsistent results (Schwab et al., 2005, Havlin et al., 2016). This may be related to the timing of niacin supplementation. When fed only post-partum, niacin does not have FFA-lowering effects (Havlin et al., 2016). However, supplementing niacin throughout the entire transition period was shown to reduce AT lipolysis effectively (Schwab et al., 2005).

Methyl donors are also nutritional supplements that when fed to transition cows limit lipid mobilization. Among these, choline and methionine are reported to reduce lipolysis in AT when fed alone (Cooke et al., 2007, Li et al., 2016) or combined (Sun et al., 2016). Chromium supplementation may promote lipogenesis in AT by enhancing the activity of the insulin receptor in adipocytes (Vincent, 2004). Nevertheless, reports on the pro-lipogenic activity of chromium are inconsistent with some studies demonstrating a NEFA lowering effect (Hayirli et al., 2001, Yasui et al., 2014) and others showing no changes in plasma lipids (McNamara and Valdez, 2005, Smith et al., 2008). The pool of available pharmacological and nutritional interventions that reduce lipolysis or enhance lipogenesis is still very limited. Exploring new drug targets that enhance insulin sensitivity and or block the lipolytic response in adipocytes will facilitate the management of transition dairy cows.

## Assessing Adipose Tissue Function During the Transition Period

Transition cow management programs often include routine measures of clinical and production parameters that can directly or indirectly evaluate AT function. Body condition score (BCS) is a good measure of subcutaneous adiposity, and the dynamics of BCS changes around parturition subjectively describes lipolysis rates. Alternatively, the use of image biomarkers obtained during ultrasound examination of AT provides an objective evaluation of BCS avoiding the variability associated with subjective visual measurements. Subcutaneous AT depth is strongly correlated with BCS evaluation when measured by trained personnel and is highly sensitive and specific in predicting plasma NEFA concentrations immediately prepartum and at calving in dairy cattle (Strieder-Barboza et al., 2015). If using subjective BCS assessment, mature cows should approach calving with a BCS of 3.0 to 3.5 and heifers with 3.25 to 3.75 as excessively thin or over-conditioned cows are more susceptible to disease.

Currently, the most common direct measure of lipolysis is plasma NEFA. In preventive herd medicine, pre and post-calving plasma NEFA values are used as early lactation disease predictors (Table 1). Similar to plasma NEFA, post-partum plasma BHB indicate NEB and predict disease risk in early lactation (Ospina et al., 2013). Lipolysis can also be evaluated at the group or individual animal level using the milk fat to milk protein percentage ratio (Table 1). Milk fat increases as plasma NEFA rise. Cows with milk fat to milk protein ratio values higher than 2 during the first week after calving are at a higher risk for developing retained fetal membranes, DA, clinical endometritis, and being culled before the end of lactation (Toni et al., 2011).

Novel biomarkers of AT function are being explored. Low concentrations of the NEFA transporters albumin and fetuin-A are associated with low lipogenic activity in AT (Strieder-Barboza et al., 2018) and may indicate a higher risk for developing fatty liver. HODEs and other oxylipids that are markers of inflammation in AT may provide disease risk information regarding AT function but still require large epidemiological studies to be validated. It is necessary to note that single biomarkers do not provide enough information to support management decisions during the transition period. However, when multiple biomarkers are analyzed together and combined with health, production, nutritional, and environmental data, biomarkers become essential for identifying metabolic problems related to extended periods of intense lipolysis.

## Conclusions

Excessive lipolysis impairs the inflammatory responses of transition dairy cows in their AT and systemically. A "lipolytic" environment around parturition exacerbates immune responses that are ineffective in clearing pathogens and affect the metabolic function. Within AT, macrophage infiltration, a key characteristic of AT remodeling is beneficial for the adaptation to the catabolic state characteristic of the transition period. However, when AT macrophage infiltration is excessive, it triggers a vicious cycle where



excessive lipolysis can exacerbate AT inflammation, which in turn further intensifies lipolytic responses.

The focus on adipose tissue biology research given by human obesity and diabetes epidemics in western countries has expanded our understanding of the role of lipid mobilization in metabolic and immune function. However, there are marked differences between ruminant and monogastric adipose organ physiology (Lalot et al., 2010), demonstrating that focused research is required on specific inflammatory and metabolic pathways linking adipocyte and immune cells function in dairy cattle.

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## SESSION NOTES

**Table 1.** Selected biomarkers of adipose tissue function in plasma and milk of transition dairy cows

Biomarker <sup>1</sup>	Suggested reference values
NEFA (Ospina et al., 2010)	< 0.50 mmol/L
BHB (Ospina et al., 2010)	1.20 mmol/L subclinical ketosis; 1.40 mmol/L clinical ketosis
Cholesterol (Kaneene et al., 1997)	1.7 to 4.3 mmol/L prepartum; 2.7 to 5.3 mmol/L postpartum
Triglycerides (Bertoni and Trevisi, 2013)	0.12 to 0.65 mmol/L
GOT/AST (Bertoni and Trevisi, 2013)	46.5 to 103 IU/L
GGT (Bertoni and Trevisi, 2013)	21 to 37 IU/L
Acetoacetate/acetone (Krogh et al., 2011)	> 10 mg/dL of acetoacetate and acetone
Albumin	3.2 to 3.7 g/dL
Fetuin A (Strieder-Barboza et al., 2018)	0.75 to 1.0 mg/mL
Milk fat: protein ratio (Toni et al., 2011)	1.00 to 1.25

<sup>1</sup> NEFA = nonesterified fatty acids; BHB = beta-hydroxybutyrate; GOT/AST = glutamic-oxaloacetic transaminase/aspartate aminotransferase; GGT = gamma-glutamyl transferase.



# New Insights on the Role of Essential Fatty Acids on Reproduction in Dairy Cattle

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## Introduction

For the past few decades, dairy researchers and the allied industry allocated major efforts to develop strategies to improve estrous detection and insemination rate in lactating cows. As a consequence, reproductive performance of dairy herds, measured by 21-d pregnancy rate, has improved in recent years (Ribeiro, 2018). Even though there is still room for further improvement in insemination rate in the average herd, the next major challenge in reproductive management of lactating cows is to find ways to consistently reduce embryo and fetal losses, and consistently increase pregnancy and calving per AI. Approximately 60% of fertilized eggs in lactating cows fail to develop to term (Ribeiro, 2018). Accordingly, pregnancy per AI remains low and stagnant for many years, and the economic burden of abortions and culling related to reproductive failures continues to lessen production sustainability of dairy herds (Ribeiro, 2018).

Pregnancy failures are ultimately caused by impaired developmental competence of the embryo or by inadequate uterine environment, which in turn are influenced by the genetics of the cow and embryo, and by environmental factors affecting ovarian and uterine biology of the cow (Ribeiro et al., 2018). Therefore, solutions for pregnancy failures will likely come from the discovery of genetic markers specifically associated with pregnancy survival and from management strategies that improve the quality of gametes and uterine environment either directly or indirectly through reduction risk factors. Advancements in nutrition management of cows during the transition and breeding periods will likely be important for achieving these goals.

Although responses are highly variable, fat supplementation improves milk production and reproduction in dairy cows (Rabiee et al. 2012; Rodney et al., 2015), which suggest that most cows are actually underfed lipids. Limited feeding of lipids is especially critical for the supply of essential fatty acids, which cannot be synthesized by bovine cells and must be supplied by the diet. In fact, the observed benefits of lipid supplementation in reproduction are often attributed to non-caloric effects of essential fatty acids (Santos et al., 2008). Nonetheless, the mechanisms by which fatty acids affect reproduction are still not completely understood. A better understanding of their role(s) in regulation of reproduction will be an important step towards development of nutraceutical strategies that consistently improve fertility in lactating cows. This review will focus on recent discoveries related to the importance of essential fatty acids to dairy cow physiology and reproduction, and their relevance to nutritional management of dairy cows.

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## Elongation of the Preimplantation Conceptus

A significant portion of pregnancy losses in dairy cows occurs during early stages of conceptus development, and include the phase of elongation. Elongation of the preimplantation conceptus is a prerequisite for maternal recognition, implantation and survival of pregnancy. It entails remarkable expansion of extraembryonic tissues along the uterine lumen in a short window of development. In cattle, elongation starts around Day 14 and, within 3 days, the conceptus grows from < 5 mm to approximately 250 mm and occupies almost the entire extension of the uterine horn (Ribeiro et al., 2018).

The exponential increase in tissue mass is explained mainly by rapid proliferation of trophoblast cells (cells of the developing placenta), which is induced by driver signals and demands substantial supply of nutrients (e.g. lipids, amino acids, sugar, nucleotides) for energy expenditures and synthesis of biomass. The required signals and nutrients are provided by the uterine histotroph (also known as uterine milk), whose secretion from endometrium and its composition are modulated by the activity of ovarian steroids and conceptus-derived molecules in endometrial cells (Ribeiro et al., 2018), and are influenced by the physiological stage and nutrition of the cow (Ribeiro, 2018).

## Role of Polyunsaturated Fatty Acids on Conceptus Elongation

The specific components of the uterine histotroph that drive conceptus elongation are not completely known. Nonetheless, the nature of these components were recently investigated by two independent studies (Barnwell et al., 2016; Ribeiro et al., 2016b) that evaluated the biology of conceptus cells at the onset of elongation, and how they sense and respond to the histotroph stimuli. Both studies revealed substantial and consistent changes in transcriptome of trophoblast cells with the onset of elongation, which suggest the nature of the elongation drivers of the uterine histotroph.

Lipid metabolism was one of the top molecular and cellular functions associated with the observed changes in gene expression in both studies. Among genes that had increased expression during elongation, some were involved with lipid uptake, lipid droplet formation, biogenesis of peroxisomes, activation, oxidation, desaturation and elongation of fatty acids, biosynthesis of phospholipids, mobilization of membrane phospholipids, biosynthesis of prostaglandins, and transport of prostaglandins and other lipids metabolites. In addition, peroxisome proliferator activated receptor gamma (PPARG) not only had transcript expression markedly increased during the onset of elongation but was also listed as an important upstream regulator of the transcriptome changes observed during elongation. In fact, PPARG is a nuclear receptor that functions as ligand-dependent transcription factor. Moreover, its transcript expression was highly correlated with the expression of other genes involved with lipid metabolism.

The PPARG have large binding pockets that interact promiscuously with multiple polyunsaturated fatty acids and their metabolites (Kliwer et al., 1997; Nagy et al., 1998). Itoh and coauthors (2008) examined crystal structures of PPARG bound to lipid ligands



and concluded that the large binding pocket of the receptor confers remarkable versatility in ligand binding and could therefore act a cellular sensor of the varying composition of the cellular pool of fatty acid ligands. Moreover, multiple omega-3 and omega-6 fatty acids were able to bind and activate PPARG.

Binding of fatty acids into the ligand pocket of PPARG causes conformational changes in the receptor that facilitates the formation of heterodimers with retinoid X receptor (**RXR**). The dimer of nuclear receptors then binds to PPAR response elements (**PPRE**) in regulatory regions of target genes to regulate gene expression. Putative PPRE were identified in regulatory regions of several genes transcriptionally regulated during the onset of conceptus elongation, which suggests a direct effect of PPARG on the abundance of the respective transcripts (Ribeiro et al., 2016b).

The hypothesis that essential fatty acids and their nuclear receptor PPARG are important for elongation of the bovine conceptus is strongly supported by an elegant study performed in sheep. Brooks et al. (2015) performed a loss-of-function study by infusing morpholino antisense oligonucleotides in the uterus of pregnant ewes from Day 7 to Day 14 after breeding using osmotic pumps. In sheep, conceptus elongation starts around day 11. Morpholino antisense oligonucleotides for PPARG resulted in the recovery of growth-retarded conceptuses, while infusion of the designed control or PPAR-delta morpholinos resulted in normally elongated conceptus on Day 14.

#### Dynamics of Fatty Acids and Oxylipids in the Uterus

Ovarian steroid hormones influence lipid metabolism in endometrial cells. The amount of lipid droplets in the epithelium fluctuates according to the phase of estrous cycle in cows (Wordinger et al. 1977) and ewes (Brinsfield and Hawk 1973), being low during metaestrus and increasing during diestrus. Brinsfield and Hawk (1973) administered 25 mg of exogenous progesterone for 5 days in ovariectomized ewes and observed an accumulation of lipids in the endometrium that was comparable to the accumulation observed in a spontaneous estrous cycle, thus concluding that progesterone was the main factor inducing the accumulation of lipids during diestrus. The mechanism by which progesterone influences the formation of lipid droplets in epithelial cells is likely caused by modulation of transcript expression of diacylglycerol O-acyltransferase 2 (**DGAT2**) and lipoprotein lipase (**LPL**), which are increased and decreased, respectively (Forde et al., 2009; 2010). The former gene is involved in synthesis of triglycerides, whereas the latter in hydrolysis of triglycerides.

Recently, we investigated the lipid content of the uterus at early-, mid-, and late-diestrus in lactating cows (Ticiani et al., 2018). Cows (n = 30) had the estrous cycle and ovulation synchronized by administration of exogenous hormones; they were blocked by parity and assigned randomly to undergo transcervical uterine flushing and biopsy on estrous cycle Days 5 (early-diestrus), 10 (mid-diestrus) or 15 (late-diestrus). Flushing and biopsy were performed in the uterine horn ipsilateral to the corpus luteum. The recovered flushing was used for analyses of lipid composition by mass spectroscopy and the tissue collected for biopsy was used for investigation of lipid droplets abundance in the

endometrium by immunohistochemistry. The abundance of lipid droplets in the endometrium increased 2-fold from Day 5 to Day 10, and another 2-fold from Day 10 to Day 15. Similarly, the concentration of fatty acids in the uterine fluid increased from Day 5 to Day 15 of the cycle. In addition, there was an enrichment of essential fatty acids and their metabolites on uterine fluid collected on Day 15 compared with Day 5 and 10. These results suggest that the uterus has a physiological mechanism to supply specific fatty acids to the uterine lumen at the time of the onset of conceptus elongation. In addition, accumulated lipids are also critical for synthesis of biomass and energy requirements of the fast growing conceptus.

#### Supplementation of Omega-3 Fatty Acids to Support Conceptus Development

Multiple studies have described benefits of supplemental omega-3 fatty acids on pregnancy success in lactating cows [reviewed by Santos et al. (2008) and Ambrose et al. (2016)]. A reduction in late pregnancy losses is the most common positive outcome, which might be associated with enhanced implantation and placental function. More recently, Sinedino et al. (2017) evaluated the effects of supplementing 100 g of an algae product rich in docosahexaenoic acid (**DHA**) in the diet of lactating cows, starting 30 days postpartum, on fatty acid composition of milk and blood phospholipids, milk production, and reproduction. Compared with non-supplemented controls, cows fed the algae product had greater incorporation of omega-3 fatty acids on milk and blood phospholipids, a 1.1 kg increase in daily milk production, greater pregnancy per artificial insemination (**AI**; 41.6 vs 30.7%) for all breedings, and fewer days open (102 vs. 124 days). Interestingly, pregnant cows in the algae group had greater gene expression of *RTP4* in peripheral blood leukocytes on Day 19 after AI, which is an indicative of enhanced elongation of the preimplantation conceptus. Altogether, these studies support a positive effect of omega-3 fatty acids on pregnancy success in lactating dairy cows, which could be mediated by better conceptus development.

#### Omega-6 Fatty Acids Are Also Important for Conceptus Development

Reducing the amount of omega-6 fatty acids, especially arachidonic acid, in the endometrium of dairy cows during the breeding period has been suggested as a strategy to minimize synthesis of luteolytic prostaglandins, to protect the corpus luteum at late diestrus, and to reduce embryonic mortality around the time of maternal recognition of pregnancy (Thatcher et al., 2006). For instance, feeding a diet rich in omega-3 fatty acids to cows increased the proportion of those fatty acids and reduced the proportion of arachidonic acid in the endometrium (Bilby et al., 2006), which resulted in a smaller prostaglandin response to an oxytocin challenge in late diestrus (Mattos et al., 2002). These associations have been suggested as a possible explanation for the improvements in reproduction observed in cows supplemented with omega-3 fatty acids (Thatcher et al., 2006). Nonetheless, the current knowledge of the biology of elongating conceptus and its high demand for arachidonic acid and prostaglandins suggest that positive effects of omega-3 supplementation on reproduction are likely caused by the higher concentration of those fatty acids per se in the endometrium and not by the reduction of omega-6 fatty acids.



Prostaglandins are produced and secreted abundantly by the developing placenta during elongation and have intracrine, autocrine and paracrine functions on conceptus and endometrium cells, which express both membrane and nuclear receptors for prostaglandins (Ribeiro et al., 2016c). Moreover, transport of prostaglandins across cell membranes is enhanced by increased expression of a prostaglandin transporter in both endometrium and conceptus cells (Ribeiro et al., 2016c). Prostaglandins are also natural ligands of PPAR $\gamma$  and, therefore, might be important for coordination of gene expression and cell biology of elongating conceptuses. Moreover, intrauterine infusion of prostaglandins in ewes during diestrus induced expression of several genes in the endometrium that are known to stimulate trophectoderm cell proliferation and migration during the elongation phase (Dorniak et al., 2011). Intrauterine infusion of meloxicam, a specific inhibitor of cyclooxygenase-2 (COX-2) activity, reduced the amount of prostaglandins in the uterine fluid of pregnant ewes and precluded conceptus elongation (Dorniak et al., 2011).

The endometrium is likely the major source of omega-6 fatty acids for the developing conceptus. In fact, Meier et al. (2011) observed a negative association ( $R^2 = 0.55$ ;  $P = 0.01$ ) between size of the conceptus and concentration of arachidonic acid in the endometrium of the gravid horn (i.e. conceptuses longer in length were associated with less endometrial arachidonic acid on Day 17 of gestation). In addition, Ribeiro et al. (2016b) reported a negative association ( $R^2 = 0.28$ ;  $P < 0.05$ ) between the concentration of arachidonic acid and prostaglandins in the uterine fluid on Day 15 of the cycle or pregnancy ( $R^2 = 0.28$ ;  $P < 0.05$ ). Thus, the presence of a healthy elongating conceptus in the uterus in late diestrus should prevent luteolytic pulses prostaglandin F $_{2\alpha}$  by endometrial cells. Also, the focus of fatty acids supplementation should shift from manipulating the production of luteolytic prostaglandin pulses to the discovery of the best fatty acid profile that supports conceptus development, which likely include both omega-3 and omega-6 fatty acids.

### Health Postpartum Is Critical for Future Fertility

Transition from the dry period to lactation is accompanied by major adjustments in the metabolism of the dairy cow to support milk synthesis. A steep increase in nutrient requirements occurs, and feed intake is inadequate to support the nutritional needs in the first weeks postpartum. Consequently, the caloric and nutrient requirements of the cow postpartum are only partially met by feed consumption, which causes extensive mobilization of nutrients from body tissues. Adipose tissue is particularly affected by reduced circulating concentrations of insulin, which up-regulate lipolytic signals for hydrolysis of stored triglycerides, increasing the availability of non-esterified fatty acids (NEFA) as energy substrate. As a consequence, lactating dairy cows usually lose large amounts of body mass postpartum, which varies according to the extent of the negative energy balance.

Extensive mobilization of body reserves during the peripartum period causes inflammation. The cellular environment of adipose tissue in cows with excessive

mobilization of fatty acids stimulates infiltration of macrophages and differentiation into a pro-inflammatory phenotype (Contreras et al., 2017). Inflammatory mediators such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-6 (IL6) are secreted from adipocytes, especially those in visceral fat, and they reduce the intracellular signaling of insulin. A large part of the NEFA mobilized from adipose tissue is composed of saturated fatty acids, mostly C16 and C18, which have the capacity to bind toll-like receptors and activate immune cells, thereby contributing to the metabolic stress postpartum (Contreras et al., 2010). The inflammation caused by the metabolic adaptation in the peripartum is further increased by microbial infections and incidence of clinical diseases commonly diagnosed in dairy cows postpartum such as metritis, mastitis, displaced abomasum, and laminitis.

Combined, excessive loss of body condition (BCS) and clinical diseases affect approximately 50% of dairy cows postpartum. Both conditions cause long-term negative effects on the reproductive biology cows, delaying the resumption of estrous cyclicity postpartum and impairing early embryonic development, including the elongation of preimplantation conceptuses (Ribeiro et al., 2016a; Ribeiro and Carvalho, 2017). The odds of pregnancy per AI is reduced approximately 30%, the odds of late pregnancy loss increases 2-fold, the odds of calving per AI is reduced 42%, the interval from calving to pregnancy is extended by weeks, and more cows are culled because of reproductive failure. Regarding conceptus elongation, cows with postpartum disorders have delayed elongation, reduced concentration of interferon-tau in the uterine fluid and expression of interferon-stimulated genes in peripheral leukocytes (Ribeiro et al., 2016a).

### Essential Fatty Acids and the Paradox of Inflammation Postpartum

Because inflammation is a common feature of postpartum problems that result in compromised fertility in the breeding period, and multiple events have additive effects on the degree of inflammation and on the degree of reduction in fertility, the process of inflammation might have a central role in mediating the long-lasting effects of postpartum disorders on reproductive biology of dairy cows. Therefore, management solutions that reduce the incidence of clinical diseases or minimize the loss of BCS postpartum or mitigate inflammation are all likely to improve reproductive performance in dairy cows. Hence, feeding essential fatty acids early postpartum can be beneficial to the following breeding period for multiple reasons: 1) it increases the energy density of the diet and, if feed intake is maintained, improves the energy balance of the cow postpartum, minimizing loss of body BCS and metabolic stress; 2) it might improve immune cell function and reduce the incidence of clinical disease postpartum; and 3) it might be used to regulate the degree and enhance the resolution of inflammation postpartum. The outcomes, however, depends on the type of fatty acids supplemented.

Reduction in the incidence of disease postpartum has been described with supplementation of omega-6 fatty acids, which enhance the production of inflammatory mediators (i.e. series-2 prostaglandins) and might improve the ability of the immune system to fight infections postpartum (Silvestre et al., 2011). Nonetheless, clear evidence exists to support that inflammation is exacerbated in many dairy cows postpartum, especially in those that develop clinical diseases and have extensive loss of BCS.



Exacerbated inflammation leads to more intense sickness behavior, a more pronounced reduction in feed intake, increased partition of energy and nutrients to support the immune system, potentially longer interval for recovery, and larger consequences for milk production and reproduction performance. Control of inflammation, on the other hand, is generally obtained with supplementation of omega-3 fatty acids. Thus, the key question is whether inflammation postpartum should be enhanced by supplementation of omega-6 fatty acids or attenuated by supplementation of omega-3 fatty acids. Ideally, management strategies should strive to improve immune cell function without increasing the degree of inflammation postpartum, which might be seen as a paradox by many.

Studies evaluating the effects of injectable anti-inflammatory treatments might provide insights on whether controlling inflammation postpartum is good or bad for the dairy cow. Administration of flunixin meglumine, a nonsteroidal anti-inflammatory drug, within 24 h of parturition increased the odds of fever (odds ratio, **OR** = 1.7), retained placenta (**OR** = 2.6), and metritis (**OR** = 1.5) postpartum, and reduced milk production (1.6 kg/d) in the first 2 weeks postpartum (Newby et al., 2009). A similar study with meloxicam, another nonsteroidal anti-inflammatory drug more selective to COX-2, failed to show differences in health and milk production postpartum (Newby et al. 2013). Nonetheless, three consecutive daily drenches of sodium salicylate (125 g/cow/day) or one bolus of meloxicam (675 mg/cow), starting 12 to 36 h after parturition, resulted in greater milk production in the entire lactation (Carpenter et al., 2016). In cows diagnosed with puerperal metritis, applying a single dose of flunixin meglumine concurrent with the antimicrobial treatment protocol did not improve clinical cure, milk production in the week after diagnosis, and posterior reproductive performance (Drillich et al., 2007). However, adding 6 doses of flunixin meglumine into the treatment protocol of puerperal metritis (2.2 mg/kg twice daily on the first 2 days and once a day in the following 2 days) reduced the incidence of fever, shortened the interval from calving to resumption of estrous cyclicity, and seemed to improve uterine involution (Amiridis et al., 2001). Moreover, adding meloxicam to the antimicrobial treatment of clinical mastitis resulted in greater pregnancy at the first AI postpartum and increased proportion of cows pregnant by Day 120 after calving compared with the control group (McDougall et al., 2016). Altogether, these studies suggest that inflammation is critical on the day of parturition and likely involved with release of fetal membranes, but the effective control of inflammation a few days after calving, especially in situations in which exacerbated inflammation is expected, might contribute to health and subsequent performance of dairy cows. Thus, a balance between inflammatory and anti-inflammatory mediators postpartum would be ideal.

A study by Greco et al. (2015) evaluated the effects of altering the ratio between omega-6 and omega-3 fatty acids in the diet of lactating cows, starting on Day 14 postpartum. Dietary treatments were isocaloric and used multiple sources of supplemental fatty acids (Ca salts of fish, safflower, and palm oils) to establish the three distinct ratios in the diet, 6:1; 5:1, and 4:1 parts of omega-6 to omega-3 fatty acids. The total amount of fatty acids supplemented was identical among treatments. Reducing the ratio from 6:1 to 4:1 - thus increasing the proportion of supplemental omega-3 fatty acids - reduced the inflammatory response to an intramammary challenge with lipopolysaccharides and increased milk production. The approach used by Greco et al.

(2015), in which the ratio of omega-6 and omega-3 fatty acids is to prioritize and not to supply only one or the other, might be a sound alternative to balance the degree of inflammation postpartum and to minimize its long-term effects on milk production and reproduction.

## Conclusions

Pregnancy losses are substantial in dairy cattle and threaten reproductive efficiency and sustainability of dairy herds. A substantial proportion of these losses occur during early stages of conceptus development, including the elongation phase. Elongation of the preimplantation conceptus is a prerequisite for maternal recognition, implantation, and survival of pregnancy. Lipids, especially essential fatty acids, in addition to be required for synthesis of biomass of the rapid growing conceptus, seem to be critical for coordination of cell biology during elongation. Thus, supplementation of essential fatty acids in the diets of lactating cows at the time of breeding has the potential to improve elongation of the conceptus and, consequently, pregnancy survival. In fact, multiple studies that evaluated the impact of supplementation of essential fatty acids, especially omega-3 fatty acids, have reported better pregnancy per AI and reduced pregnancy losses. The outcomes, however, remain variable, and a better understanding of the role of essential fatty acids on uterine physiology will contribute to the development of better and more consistent strategies to minimize early and late pregnancy losses.

Clinical diseases and excessive loss of body reserves are prevalent problems in postpartum dairy cows and represent important risk factors for pregnancy losses in the subsequent breeding period. Exacerbated inflammation postpartum seems to be a common feature of postpartum conditions that affect fertility of cows. Thus, supplementation of essential fatty acids early postpartum might also contribute to reproduction of dairy cows by increasing energy intake and minimizing loss of BCS, by improving immune cell function and minimizing the incidence of clinical disease, or by controlling the degree of inflammation postpartum and enhancing the recovery from clinical and metabolic problems postpartum. Finding a balance between pro-inflammatory omega-6 and anti-inflammatory omega-3 fatty acids seems to be a sound strategy to optimize the transition from the dry period to lactation, which substantially impacts performance in the entire lactation. A major challenge in nutrition management of transition cows is to find strategies that improve immune cell function without causing exacerbated inflammation postpartum, which continues to be seen as a contradiction by many dairy researchers.

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## SESSION NOTES



## Technologies for Improving Fiber Utilization

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### Introduction

Forages typically account for 40 to 100 % of the ration of dairy cows and are vital for maintaining animal productivity and health. The high fiber content of forages is the main nutritional factor that differentiates them from concentrates and results in a relatively lower energy value. Nevertheless, fiber plays a fundamentally important role in ruminant livestock production, health, and welfare. In addition to being an important energy source, it stimulates chewing and salivation, rumination, gut motility, and health, buffers ruminal acidosis, regulates feed intake, produces milk fat precursors and is the structural basis of the scaffolding of the ruminal raft, which is vital for digestion of solid feed particles in the rumen. Cellulose and hemicellulose – the main components of fiber – are intrinsically ruminally digestible. However, their close association with lignin and hydroxycinnamic acids like ferulic acid in the plant cell wall is the greatest hindrance to complete digestion of feeds, particularly forages and byproducts, and to utilization of the nutrients and energy they contain. The degree of association with lignin and hydroxycinnamic acids and various plant anatomical features differentiate digestible from indigestible fiber. This paper describes the strategic importance of increasing forage fiber utilization and then discusses the efficacy and mode of action and benefits and disadvantages of different technologies for improving fiber digestion.

### Importance of Increasing Fiber Digestion

It is critically important to increase fiber digestion for productivity, profitability and environmental reasons. Incomplete fiber digestion reduces the profitability of dairy production by limiting intake and hence, animal productivity, and increasing manure production. A 1-unit increase in forage NDF digestibility (NDFD) is associated with 0.17 and 0.25 kg/d increases in DMI and milk production, respectively (Oba and Allen, 1999). In addition, in perennial ryegrass (*L. perenne*), a 5–6 % increase in digestibility was estimated to increase milk production by up to 27% (Smith et al., 1998). Consequently, each percentage unit increase in lignin concentration in forage cell walls severely constrains DMI and milk production.

A second reason to increase fiber digestion is to increase energy supply from fibrous feeds that are not consumed by humans. Grains are high in energy and various

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processes have been developed for increasing the efficiency of energy extraction from such feeds for ruminants and non-ruminants. However, the growing demand for grains due to competition with the non-ruminant feed, biofuel and human food sectors, results in considerable price hikes and volatility. Fibrous feeds for ruminants are less subject to such competing demands but their recalcitrant lignocellulose matrix reduces the availability of the energy they contain, necessitating effective strategies for increasing the rate and efficiency of utilization of forage fiber and the energy therein.

It is equally necessary to increase fiber digestion for environmental reasons. Compared to that from starch, ruminal fermentation of fiber-derived hexoses generates more hydrogen ions that reduce carbon dioxide to methane. Consequently, fibrous feed fermentation results in greater production of methane and less energy supply than concentrate feeds. In addition to being a significant drain on energy supply to the cow, enteric methane production is a significant contributor to greenhouse gas emissions. In addition, enteric methane is the main source of agriculture-related methane emissions also, which has resulted in advocacy of vegan diets for environmental reasons (Poore and Nemecek, 2018) and cessation of livestock production, despite the critical role livestock and livestock products play in human nutrition, income generation and livelihoods (Adesogan et al., 2018) or the fact that because ruminants convert forages that humans cannot consume into high quality food protein, only 14% of feed dry matter ingested by livestock is edible to humans (Mottet et al., 2017). Consequently, it is of paramount importance to increase forage fiber digestion to enhance animal productivity and environmental stewardship of livestock farming.

Various animal, plant, and environmental factors that modulate the intake and digestibility of forage fiber have been described in excellent reviews (Galyean and Goetsch, 1993; Fales and Fritz, 2007). The main plant-based factors affecting the digestibility and intake of forage fiber are: 1) chemical composition of tissues in organs, 2) tissue type and proportion within organs, and 3) organ type and proportion in plants. The interplay between these factors has a major effect on the intake and digestion of nutrients by ruminants (Jung, 2012), with chemical composition becoming more predominant than the other factors with increasing levels of mechanical processing.

### Strategies to Increase Forage Fiber Digestion

**Mechanical Processing.** Mechanical processing is a crucial complementary step in forage production due to its impact on forage physical properties that cause gut fill and limit feed intake. Consequently, numerous studies have examined the influence of mechanical processing on particle size measures, typically the chop length of hay, straw, or silage or the particle size distribution of diets for lactating cows. Forage particle size is critically important in dairy cattle diets, which must contain sufficient physically effective NDF (peNDF; Hall and Mertens, 2017) – a combination of both physical (i.e. particle size) and chemical (i.e. NDF concentration) fiber characteristics (Mertens, 1997) – to stimulate chewing and salivation and reduce gut fill, without reducing digestibility. In this context, grinding of forages or byproducts will not be discussed because it removes the physical effectiveness of fiber.



**Chopping.** Despite the undeniable benefits of coarser forage particles on ruminal mat formation, chewing activity, digestion, and milk fat content (Allen, 1997; Mertens, 1997), long forage particles may limit intake through reduced ruminal passage rate and increased fill (Mertens, 1987). Furthermore, they promote dietary sorting (Leonardi and Armentano, 2003), and enhance the time spent consuming a meal (Grant and Ferraretto, 2018). Although particle size can be manipulated to enhance fiber digestibility, research findings have been inconsistent, and the outcome is not related to alterations in the chemical composition of forage fiber. A meta-analysis of published studies (Ferraretto and Shaver, 2012b) reported that digestibility of dietary NDF, DMI, and milk production were not altered by chop length of corn silage. This should not be surprising as fiber digestion is influenced by many factors and the combination of the benefits of long or short forage particles may be countered by the disadvantages. For instance, short forage particles have greater surface area for bacterial attachment, which may enhance forage digestibility despite their faster passage rate (Johnson et al., 1999). In contrast, coarse particles are retained for longer periods in the rumen and require more chewing leading to greater ruminal pH (Allen, 1997), which is conducive for cellulolytic bacteria and forage digestion in general.

**Shredding.** Shredding ensiled whole-plant corn at harvest is an effective method to alter the physical characteristics of the silage. A recently developed form of silage, called corn shredlage, is produced when corn forage is harvested with a self-propelled forage harvester fitted with cross-grooved crop-processing rolls set at approximately 20% greater roll speed differential and chopped at a greater theoretical length of cut (22 to 26 mm) than the norm. Despite the longer chop length used, the shredding process causes greater damage to coarse stover particles and kernels than conventional harvesting. Compared to conventionally processed silage, yields of 3.5% FCM and actual milk were increased by 1.0 and 1.5 kg/cow per day when whole-plant corn was harvested as corn shredlage using either conventional (Ferraretto and Shaver, 2012a) or brown midrib (BMR; Vanderwerff et al., 2015) hybrids, respectively. These results were attributed to the greater kernel breakage obtained by shredding whole-plant corn and the corresponding improvements in ruminal in situ and total tract in vivo starch digestibility (Ferraretto and Shaver, 2012a; Vanderwerff et al., 2015). Surprisingly, despite what appeared to be more thorough damage to the fibrous portion of the forage, Vanderwerff et al. (2015) reported that total tract NDFD was 2 percentage units lower when cows were fed corn shredlage instead of conventionally processed corn silage. These authors associated this response with the negative effects of the greater digestibility of shredlage starch on total tract NDFD. This premise was supported by the fact that ruminal in situ NDF digestibility of undried and unground corn silage samples did not differ among treatments. Finally, near-infrared reflectance spectroscopy-predicted 30-h NDFD was lower in corn shredlage (55.0 vs. 53.4% of NDF) compared to conventionally processed corn silage in an assessment of 3,900 commercial samples (Ferraretto et al., 2018). Although the benefits of harvesting corn silage with a shredlage processor are undeniable, some factors must be considered when evaluating the cost effectiveness. In addition to the costs associated with acquiring the processor (or a new self-propelled forage harvester), other factors such as changes in fuel usage and roll wear must be

considered as they may differ from those involved in conventional processing. To our knowledge, this information is unavailable in the literature and should be the focus of future research.

**Pelleting.** In addition to the aforementioned effects of particle size, pelleting may enhance handling, storage and transportation (Bonfante et al., 2016), and it enhances the use of certain bulky forage or crop residues as livestock feeds (Mani et al., 2006). The use of forage pellets, however, is not a new concept. For example, Clifton et al. (1967) evaluated coastal Bermudagrass (*Cynodon dactylon* (L.) Pers.) fed as either silage or pellets. Cows fed forage pellets had greater intake but did not have greater animal performance. Caution is needed when forages are fed as pellets to due to the risk of acidosis from the reduced saliva production and resulting reduction in ruminal acid buffering caused by pelleting, but the effect may depend on the production stage of the cows. Bonfante et al. (2016) fed a pelleted TMR to growing heifers and did not observe adverse effects on ruminal health, though the authors advocated examining the pellets for longer feeding periods. Total tract digestibility of the potentially digestible NDF fraction was reduced, presumably due to reduced ruminal retention time. This suggests that using pelleted TMR for growing heifers may not adversely affect rumen health, but caution is needed to ensure digestibility is not reduced. In contrast, when alfalfa (*Medicago sativa* L.) pellets were substituted for alfalfa hay to induce subacute ruminal acidosis in dairy cows (Khafipour et al., 2009; 8% - unit increments from 50 to 10%, DM basis) over 6 weeks without altering forage to concentrate ratio and starch concentration, a gradual increase in consumption of pellets instead of hay was evident but yields of milk and milk fat and ruminal pH decreased in a linear manner. The latter results emphasize that pelleting TMR for lactating cows can be detrimental and reinforces the need to account for peNDF during diet formulation.

## Genetic Improvement

**Brown-midrib mutants.** Improvements to fiber digestibility of forages are often accomplished by reducing lignin or indigestible NDF concentrations (Grant and Ferraretto, 2018). Brown midrib mutant forages (e.g., corn and sorghum) consistently have lower lignin concentrations compared to conventional forages (Sattler et al., 2010) resulting in greater milk production when the BMR forages are fed. In this context, several studies have reported greater DMI, passage rate, and rate of NDF digestion in cows fed BMR compared to conventional corn silage (Oba and Allen, 2000; Ebling and Kung, 2004). In a meta-analysis of published studies, Ferraretto and Shaver (2015) reported increases in total tract NDFD (44.8 vs. 42.3% of intake), DMI (24.9 vs. 24.0 kg/d), yields of milk (38.7 vs. 37.2 kg/d) and protein (1.18 vs. 1.13) for cows fed BMR diets instead of conventional corn silage diets. These benefits are associated with lower rumen gut fill as conventional forage-based diets may have lower rates of passage and digestion, causing physical constraints in the rumen (Allen, 1996) that limit intake.

As for corn, BMR sorghum (*Sorghum bicolor*) has lower lignin concentration and greater fiber digestibility compared to conventional sorghum (Sattler et al., 2010). A meta-analysis by Sánchez-Duarte et al. (2019) compared conventional to BMR sorghum silage



(BMRSS) in diets for dairy cows and revealed that cows fed BMRSS had greater intake (+0.8 kg/d), milk production (+1.6 kg/d) and milk fat concentration (+0.09%-units) than cows fed conventional sorghum. In addition, when compared with conventional corn silage, cows fed BMRSS had greater milk fat (+0.10%-units) but lower milk protein (-0.06%-units) concentrations. No differences in intake and milk yield were observed. Such BMR hybrids would be particularly desirable in areas or situations unsuitable for corn production. Some studies have shown that lodging can be a problem for some BMR sorghum hybrids particularly when sown at high seeding rates (Pedersen et al., 2005), but the incidence may be reduced by increasing plant spacing or planting brachytic dwarf hybrids that are less prone to lodging (Bernard and Tao, 2015).

Genetic improvement is resulting in BMR hybrids that are higher yielding than earlier hybrids. Nevertheless, it is important to account for lower yields of certain BMR hybrids than conventional hybrids when deciding on which hybrid to grow. Such lower yields may be outweighed by the improved animal performance from BMR hybrids but the magnitude of the improvements may vary from farm to farm based on the prevailing conditions. Producers should consider establishing guidelines for using BMR hybrids such as feeding them to high-producing cows in early lactation while feeding less digestible conventional hybrids to cows in mid-to-late lactation. Such guidelines should be based on recommendations of animal nutritionists and agronomists who are familiar with the prevailing conditions on the dairy farm.

**Reduced-lignin alfalfa.** Feeding reduced-lignin alfalfa to dairy cows has been studied for over a decade. Guo et al. (2001) examined the lignin concentration and IVNDFD of 6 independent transgenic alfalfa lines with reduced lignin concentration compared to control lines (non-transgenic) and reported a range from 13 to 29% in lignin concentration. Furthermore, they observed an increase of 8% in IVNDFD for one of these transgenic lines compared to its isogenic counterpart. Mertens and McCaslin (2008) fed transgenic alfalfa hay with reduced lignin concentration (5.3 vs. 5.8% of DM) to young lambs and observed greater NDF intake (1.6 vs. 1.42% of BW/d) and digestibility (57.5 vs. 49.1% of NDF intake) compared to a non-transgenic line. When this same transgenic alfalfa variety was fed to dairy cows by Weakley et al. (2008), total tract NDFD was greater for cows fed transgenic compared to non-transgenic alfalfa but no differences in DMI, milk yield or milk fat concentration were observed. Li et al. (2015) tested effects of 2 transgenic alfalfa cultivars (Roundup-ready vs. Roundup-ready low-lignin) and reported greater in vitro total tract NDFD for the low-lignin alfalfa. This response was primarily driven by alterations in the NDF to lignin ratio as lower NDF (30.1 vs. 31.6% of DM) but similar lignin (5.6 vs. 5.5% of DM) concentrations were reported for the Roundup-ready conventional versus Roundup-ready low-lignin cultivar. However, no peer-reviewed dairy cow feeding studies on low-lignin alfalfa was found, so caution is required when interpreting these results; further studies evaluating responses of dairy cows are warranted. As for BMR hybrids, it is important to account for potential variations in yields and prices when choosing between reduced-lignin alfalfa and conventional varieties.

## Chemical Treatment

**Alkali treatment.** Alkali treatments break hemicellulose-lignin and lignocellulose bonds, hydrolyzing uronic and acetic acid esters, and disrupting cellulose crystallinity by inducing cellulose swelling (Jung and Deetz, 1993). These processes increase cell wall degradability and enable ruminal microorganisms to attack the structural carbohydrates and increase degradation of hemicellulose and cellulose (Jung and Deetz, 1993; Sun et al., 1995). Additionally, alkali treatment has potential to degrade lignin, thereby increasing its water solubility and allowing it to be removed from the cell wall (Chesson, 1988). Various alkalis including ammonia, sodium hydroxide (NaOH), calcium oxide (CaO) and calcium hydroxide (Ca(OH)<sub>2</sub>) have been used to increase fiber digestion and hence nutritive value of low quality forages, particularly crop residues (Singh and Klopfenstein, 1998). However, their widespread adoption to improve forage quality has been hindered by factors like the cost of application, the hazardous nature, or their corrosiveness.

**Ammoniation.** Improves forage digestibility by hydrolyzing linkages between lignin and structural polysaccharides (Dean et al., 2008). Ammoniation of low quality forages like bermudagrass hay (Dean et al., 2008; Krueger et al., 2008), Bahiagrass hay (Krueger et al., 2008), cereal straws including barley, wheat, and oat (Horton and Steacy, 1979) has resulted in improved intake, increased DM and NDF digestibility, and N concentration, and improved milk production (Kendall et al., 2009). However, the effects of feeding ammoniated forages on lactation performance by cattle are not consistent (Brown et al., 1992). Also, ammoniation has not gained widespread commercial acceptance due to its high cost, and caustic effect of the alkali when inhaled or ingested excessively by humans and animals (Krueger, 2006). Urea treatment is a safer and easier method of ammoniation that poses far less handling and safety risks (Sundstol and Coxworth, 1984). In addition, it is easy to transport and store. However, the amount of urease activity and moisture content of forages determine the efficacy of urea treatment, as both are required for the formation of ammonia from urea.

**Ammonia-fiber expansion.** The ammonia-fiber expansion (AFEX) is an alternative to direct ammoniation that combines chemical and physical treatments. The method involves ammoniating low quality forages at high temperature and pressure, with subsequent pressure release and ammonia removal (Campbell et al., 2013; Griffith et al., 2016) or recycling. Recently, Griffith et al. (2016) reported 35 and 27% greater in vitro dry matter digestibility (IVDMD) and IVNDFD due to AFEX treatment of barley straw. Mor et al. (2018) reported improved nutrient digestibility (DM, OM, CP, NDF, and ADF) of AFEX-treated wheat straw. However, acetamide, a co-product of the AFEX treatment (Weimer et al., 1986) may remain with the treated biomass. Early research indicates that ruminal accumulation of acetamide from AFEX treatment is transient in the rumen because certain ruminal bacteria can grow on the amide (Mor and Mok, 2018). More research on the effects and fate of residual acetamide in cattle fed AFEX-treated forages is needed to ensure that meat and milk are not contaminated.

**Sodium hydroxide.** Sodium hydroxide treatment originally entailed soaking forage with the dilute alkali for several days followed by washing to remove unreacted residues (Jackson, 1977). This was effective at improving in vitro OM digestibility of a low-quality forages like rye straw from 46 to 71% (Sundstol, 1988), but it contributes to



environmental pollution via the effluent. The dry method of NaOH treatment involves spraying dilute NaOH onto the forage without rinsing prior to feeding. Treating rice straw with 4% NaOH via the dry method improved net energy value and increased DMI, and growth performance in steers and feeder lambs (Garrett et al., 1974). Similarly, NaOH treatment (4% DM basis) of corn stalks wetted with 50% moisture from added water increased OM digestibility by 20% compared to untreated stalks (Klopfenstein et al., 1972). The main advantage of the dry method is that it is less labor intensive and issues with wastewater pollution are avoided. However, because excess NaOH is not rinsed off, there are greater chances of toxicity if forage samples are not uniformly treated. Like ammonia, NaOH is caustic and hazardous.

**Calcium oxide or calcium hydroxide.** Alternatives to ammonia and NaOH are less hazardous alkalis such as CaO and Ca(OH)<sub>2</sub>. Wanapat et al. (2009) observed greater DMD and 3.5% FCM production by dairy cows fed rice straw treated with a combination of urea (2.2%) and Ca(OH)<sub>2</sub> (2.2%). Chaudhry (1998) treated wheat straw with CaO and reported greater OM, NDF and ADF digestibility. Similarly, Shreck et al. (2015) reported greater DMI, feed efficiency, and average daily gain in beef steers with diets containing 5% CaO treated-corn stover or wheat straw compared to diets using the untreated forages.

**Acid treatment.** Acid hydrolysis for pretreatment of lignocellulosic materials is hydrolyzes hemicellulose, decreases cellulose crystallinity, and increases the porosity of treated biomass (Sun and Cheng, 2005). To foster ease of handling and cost-effectiveness, dilute acid treatment is preferred. Torget et al. (1990) treated switchgrass with dilute sulfuric acid (H<sub>2</sub>SO<sub>4</sub>; 0.45-0.50%, v/v) and reported 95% xylan hydrolysis and concomitant improvement in cellulose digestibility. Similar results were observed with dilute H<sub>2</sub>SO<sub>4</sub> pretreatment of corn cobs and corn stover (Torget et al., 1991). Acid hydrolysis can also improve subsequent enzyme-mediated increases in cell wall digestibility by increasing the pore size of the treated material as reported for corn stover (Ishizawa et al., 2007). However, acid pretreatment is not widely used of the cost, health hazards and corrosive nature of the acids.

Studies have also reported use of inorganic (H<sub>2</sub>SO<sub>4</sub>) and organic acids (formic acid) as silage preservatives (O'Kiely et al., 1989; Henderson, 1993). Sulfuric acid reduces the pH of forage thereby inhibiting the activity of undesirable bacteria such as enterobacteria and clostridia, and stimulating lactic acid bacteria; however, the effects on animal performance are not promising (O'Kiely et al., 1989). Organic acids, in particular formic acid, induce antibacterial activity and restrict the activity of lactic acid-producing bacteria thereby conserving water soluble carbohydrates for animals (Bosch et al., 1988). Studies have reported decreased acetic acid, lactic acid, and ammonia-N concentrations along with greater sugar concentrations with formic acid-treated alfalfa (Nagel and Broderick, 1992) or ryegrass (Mayne, 1993), compared to the control silage. The effects of feeding formic acid treated silage on animal performance are inconsistent. The use of acids as silage preservatives has declined due to their corrosive effects on machinery and potential health hazards for humans (Lorenzo and Kiely, 2008). Ammonium tetraformate is a buffered form of formic acid, which is less corrosive in nature and easier to handle.

Broderick et al. (2007) fed ammonium tetraformate-treated alfalfa silage to lactating dairy cows and reported greater DMI, and yields of milk, milk protein, FCM and greater N-efficiency compared to untreated alfalfa silage.

## Exogenous Fibrolytic Enzymes

**Cellulase-xylanase enzymes.** Limited understanding of the composition and mode of action of exogenous fibrolytic enzymes (EFE) has restricted the development of effective EFE preparations that consistently improve fiber digestion and the performance of cattle (Beauchemin and Holtshausen, 2010; Adesogan et al., 2014). The effects of EFE on forage nutritive value are influenced by various factors including the dose, activity and composition (Eun and Beauchemin, 2007), proteomic profile (Romero et al., 2015a), prevailing pH and temperature (Arriola et al., 2011), presence of metal ion cofactors (Ca<sup>2+</sup>, Co<sup>2+</sup>, Fe<sup>2+</sup>, Mg<sup>2+</sup>, and Mn<sup>2+</sup>) (Romero et al., 2015b), animal performance level (Schingoethe et al., 1999), and the dietary fraction to which the EFE is applied (Dean et al., 2013).

The effects of EFE in ruminant diets can be classified as pre-ingestive, ruminal, and post-ruminal (McAllister et al., 2001). When EFE are applied to fibrous substrates before feeding, fiber hydrolysis can be observed as partial solubilization of NDF and ADF and release of sugars and free or monomeric hydroxycinnamic acids (Krueger et al., 2008; Romero et al., 2015c). These factors may contribute to improvements in in vitro fiber digestibility (Romero et al., 2015a) and microbial growth (Forsberg et al., 2000). Adding EFE to the diet increases the hydrolytic capacity of the rumen mainly due to increased bacterial attachment (Wang et al., 2001) and stimulation of ruminal microbial populations (Nsereko et al., 2002). Furthermore, Morgavi et al. (2000) showed that synergism between EFE and ruminal microbes enhanced ruminal cellulose, xylan and corn silage digestion. Adding EFE may also increase the hydrolytic capacity of the rumen by adding complementary enzyme activities that are absent. For instance, rumen metagenomic and metatranscriptomic studies have shown that the glycoside hydrolase family GH7 is absent in the rumen but is present in certain aerobic microorganisms (Dai et al., 2015).

Application of EFE often increases fiber hydrolysis and NDFD of forages, which partially explains their ability to improve animal performance. A recent meta-analysis of published studies reported that EFE application to dairy cow diets resulted in an increase in milk yield (0.83 kg/d) and this was attributed to a tendency for EFE to improve NDF and DM digestibility (Arriola et al., 2017). This meta-analysis also reported that application to the TMR instead of the concentrate or forage tended to improve milk protein concentration. Another recent meta-analysis reported an increase in milk yield (1.9 kg/d) when cows were fed enzyme-treated diets containing high forage to concentrate ratios (≥ 50%), but no increase occurred when diets with low forage to concentrate ratios (< 50%) were fed (Tirado-Gonzalez et al., 2017). The latter study reported also that cellulase-xylanase enzyme treatment of high-forage, legume-based diets increased milk production by cows (2.3 kg/d) as did xylanase treatment of high-forage grass-based diets (3.1 kg/d).



Though the meta-analyses cited above indicate that overall effects of EFE on fiber digestion and milk yield by dairy cows are positive, the results of individual studies have been variable. This is partly because of inadequate understanding of enzyme nomenclature and activity, which results in some ineffective preparations (Adesogan et al., 2014), degradation of enzymes and loss of their activities in the rumen (Colombatto and Beauchemin, 2003; Arriola et al., 2017), adaptation of enzymes developed for paper, textile and other applications for ruminant nutrition (Beauchemin et al., 2003; Adesogan et al., 2014) and formulation of enzyme products that do not complement ruminal enzyme activities (Ribeiro et al., 2018). Recent approaches like proteomics, metagenomics and metatranscriptomics, are providing a better understanding of the structure, interaction, and functions of the ruminal microbial community (Meale et al., 2014). The knowledge generated by these new techniques should be exploited in formulating enzyme preparations that will persist in the rumen and effectively and consistently improve fiber digestion.

**Esterase and etherase enzymes.** When ferulic acid cross-links arabinoxylans and lignin via ester and ether linkages, in plant cell wall, the extent of digestion is reduced dramatically (Jung and Deetz, 1993). Jung and Allen (1995) hypothesized that the ester portion of the ferulic acid bridge is not available to enzymes because the lignin polymer is in such close proximity, impeding substrate attachment. Ferulic and p-coumaric acid esterases have been used recently to increase the potency of EFE in ruminant diets (Beauchemin et al., 2003; Krueger et al., 2008).

Etherase enzymes are required to hydrolyze ether linkages and release ether-linked ferulic acid from cell walls but they are produced rarely by fungi and are not present in the rumen environment. Mathieu et al. (2013) reported no  $\beta$ -etherase activity from 26 fungal strains (including *Humicola grisea*, *Aspergillus* sp. and *Trichoderma viride*) within 3 ecological groups (white, brown, and soft - rot fungi) cultured with Tien and Kirk medium and supplemented with or without sawdust. The authors concluded that cleavage of  $\beta$ -aryl ether linkage by extracellular  $\beta$ -etherase is a rare and nonessential activity among wood-decaying fungi.

**Bacterial inoculants.** Applying EFE with microbial inoculants to forages at ensiling is beneficial as they may hydrolyze plant cell walls into sugars that serve as fermentation substrates, thus improving silage fermentation, nutrient preservation and utilization of the silage by animals (Muck and Bolsen, 1991). Consequently, some silage inoculant preparations contain fibrolytic enzymes, mainly cellulases or xylanases, and some studies have reported increased silage NDFD due to application of such products (Filya and Sucu, 2010; Queiroz et al., 2012).

Certain inoculants contain bacteria that secrete fibrolytic enzymes including cellulases, xylanases, and ferulic acid esterase (FAE) that may contribute to increased fiber digestion. Addah et al. (2011) reported that using a mixed bacterial culture containing *L. buchneri* LN4017 that produces FAE, and contains *L. plantarum* and *L. casei*, increased in situ NDF disappearance after 24 and 48 h of incubation by 40.5 and 14.5 %, respectively. Unfortunately, the enzyme activities or enzyme-secreting ability of inoculant

bacteria are rarely declared on inoculant labels. Nevertheless, in recent meta-analyses, although no effects on NDFD were observed when bacterial homofermentative and facultative heterofermentative inoculants were applied to forages, milk yield was improved (Oliveira et al., 2017). More information is needed on the enzyme activities produced by inoculant bacteria, as this may lead to development of inoculants that are more potent at increasing fiber digestion.

**Expansins.** Expansins and expansin-like proteins are a recently discovered group of non-hydrolytic proteins with the unique ability to induce cell-wall relaxation or loosening (Cosgrove, 2000). They are relatively small proteins (between ~26 to 28 kDa) with disruptive activity that weakens cellulose fibers thereby enhancing accessibility and hydrolysis by cellulases and hemicellulases (Kim et al., 2009). Perhaps the most remarkable characteristic of expansins and expansin-like proteins is their ability to synergize with EFE to increase hydrolysis of cellulose and hemicellulose (Kim et al., 2009; Bunternngsook et al., 2015; Liu et al., 2015). Previous studies have demonstrated that synergistic effects between BsEXLX1 and exogenous fibrolytic enzymes (EFE) increased hydrolysis of cellulose and hemicellulose more than 5-fold compared to EFE alone (Kim et al., 2009). Recently, it was demonstrated that BsEXLX1 has greater affinity towards substrates with high concentrations of lignin (Kim et al., 2013), which further confirms that they may be particularly effective at increasing the efficacy of EFE at digesting the forage lignocellulose complex, particularly in C<sub>4</sub> grasses and legumes, which tend to be more lignified than C<sub>3</sub> grasses.

The recombinant expression of expansin and expansin-like protein is currently the only viable method to study these proteins due to lack of commercially available products. Bacterial expansin-like proteins (**BsEXLX1**) from *Bacillus subtilis* have been used as the gold standard to study the disruptive effects of expansins and expansin-like proteins on hydrolysis of cellulose. Preliminary studies have shown greater IVDMD and IVNDFD of bermudagrass silage by approximately 4% and 16%, respectively by applying both additives instead of the EFE alone (Pech-Cervantes et al., 2017). In contrast, no synergistic increases in IVDMD or IVNDFD were observed with corn silage (Pech-Cervantes et al., 2017).

**Live yeast, yeast culture and yeast fermentation products.** Yeast products include live yeast, yeast culture and yeast fermentation products that can be produced from different strains of *Saccharomyces cerevisiae*. Various studies have shown that yeast products improved fiber utilization and animal performance (Ferraretto et al., 2012; Jiang et al., 2017a). However, some studies have shown that they did not improve nutrient digestibility (Ouellet and Chiquette, 2016), dairy cow performance (Ferraretto et al., 2012), or ruminal fermentation and microbiome composition (Bayat et al., 2015). A meta-analysis by Desnoyers et al. (2009) showed that adding live yeast to dairy cow diets increased OM digestibility by 0.8 percentage-units, DMI by 0.44 kg/d, and milk yield by 1.2 kg/d by dairy cows. Similarly, in another meta-analysis by Poppy et al. (2012), yeast culture addition to the diet increased milk and milk fat and protein yields by 1.18, 0.06, and 0.03 kg/d. Increases in milk production by yeast supplementation may be due to improvement in fiber utilization.



Yeast supplementation has decreased lactate production and enhanced lactate utilization (Lynch and Martin, 2002); thereby stabilizing ruminal pH and increasing NDFD (Marden et al., 2008). In addition, live yeasts have the ability to scavenge O<sub>2</sub> and reduce the redox potential of ruminal fluid (Newbold et al., 1996). These changes supposedly make the ruminal environment more conducive for the growth of anaerobic microorganisms including cellulolytic bacteria (Newbold et al., 1996; Marden et al., 2008). Additionally, yeast product supplementation may provide soluble growth factors such as vitamin B, amino acids, and organic acids that are beneficial for the growth of major cellulolytic bacteria (Callaway and Martin, 1997; Jiang et al., 2017 a, b).

Overall, supplementation with live yeast or yeast culture increased milk yield by dairy cows but the magnitude of the response varies with the lactation stage of cows, the yeast product, diet composition and stressors on the cow. Therefore, future studies should aim to optimize yeast products to achieve consistent improvements in fiber digestion and animal performance over a wide range of conditions. In addition, research should identify the relative importance of the main active ingredients of yeast products, particularly yeast cultures or fermentation products to determine their mode of action.

**White-rot fungi** The white-rot fungi achieve lignin depolymerization through the activity of their ligninolytic enzymes, which include lignin peroxidase, manganese peroxidase, versatile peroxidase, laccase, and H<sub>2</sub>O<sub>2</sub>-forming enzymes (such as (methyl) glyoxal oxidase and aryl alcohol oxidase) (Wong, 2009; Sindhu et al., 2016). In addition, white-rot fungi also use extracellular reactive oxygen species, which may initiate lignocellulose decay, as lignocellulose-degrading enzymes are too large to penetrate an intact cell wall (Srebotnik et al., 1988; Blanchette et al., 1997). In addition to ligninolytic enzymes, certain white-rot fungi also produce cellulose-degrading enzymes ( $\beta$ -glucosidase, cellobiohydrolase, and  $\beta$ -xylosidase) (Vrsanska et al., 2016), resulting in simultaneous degradation of lignin and cellulose components by several strains (*Trametes versicolor*, *Heterobasidium annosum*, and *Irpelex lacteus*). Consequently, white-rot fungi can improve digestibility and nutritive value of low-quality forages such as wheat straw and bermudagrass (Akin et al., 1993; Nayan et al., 2018). Tuyen et al. (2012) reported that 9 of 11 species of white-rot fungi increased in vitro NDF and ADF digestibilities of wheat straw. However, excessive carbohydrate degradation is one of the main drawbacks to using some strains of white-rot fungi to improve utilization of fiber by ruminants (Wong, 2009; Sarnklong et al., 2010). Nonetheless, considerable variability in fiber degradation potential exists among different strains of the same species.

Few studies have involved feeding white rot fungi to animals. A notable exception is the study of Fazaeli et al. (2004) in which treatment of wheat straw with a lignin-selective strain, *Pleurotus ostreatus* (P-41) increased DMI (12.2 vs. 10.6 kg/d), DM digestibility (58.8 vs 52.3 %), NDFD (42.3 vs. 34.3 %), milk yield (9 vs. 7.5 kg/d), and BW gain (743 vs. 272 g/d) of dairy cattle in late lactation. Similarly, Shrivastava et al. (2014) reported greater DMI (per kg metabolic BW), DM digestibility (57.82 vs. 52.07 %), NDFD

(53.3 vs. 45.8 %) and 50 g/d higher average BW gain, when buffalo calves were fed wheat straw treated with *Crinipellis* sp. RCK-1 instead of untreated wheat straw.

Despite these positive responses, white-rot fungi are not widely used for increasing ruminant fiber digestion due to the long pretreatment time required (van Kuijk et al., 2015) and more importantly, the risk of degradation of digestible carbohydrates, thus reducing the nutrient content and digestibility of the residual forage. While careful strain selection can help minimize such problems, it should be noted that laccase, is a potential inhibitor of cellulase activity (Moreno et al., 2012; Yingjie et al., 2018) and fungal delignification is an aerobic process (van Kuijk et al., 2015) that does not occur in the anerobic rumen.

**Brown-rot fungi.** Brown-rot fungi can degrade lignocellulose polysaccharides by supposedly modifying rather than removing lignin (Highley, 1991) and producing enzymes that selectively depolymerize cellulose and hemicellulose, leaving a brown-colored rot (Gao et al., 2012). The modifications to lignin include demethylation, hydroxylation, and side chain oxidation (Arantes et al., 2012; Martinez et al. 2011).

Several studies have used brown-rot fungi to pretreat biomass for biofuel production, but few animal nutrition studies have used them to increase fiber utilization in ruminant diets. Gao et al. (2012), pretreated corn stover with different strains of white- and brown- rot fungi and reported that the greatest conversion of cellulose to glucose occurred with a strain of brown-rot fungi, *G. trabeum* (KU-41), after 20 days of pretreatment. These authors reported 32.0 and 31.4% conversion of xylan to xylose with 2 strains of *G. trabeum*, KU-41 and NBRC6430, respectively, compared to 11.2% for the control treatment, after 48 h of enzymatic hydrolysis. El-Banna et al. (2010) reported that in vivo digestibility of CP, NDF, ADF, hemicellulose and cellulose of sugarcane bagasse treated with the brown rot fungi, *Trichoderma reesei* (F-418), were increased compared to the untreated control, when fed to sheep. The authors reported that incubation of crop residues (bean straw, rice straw, corn stalk and sugarcane bagasse) with *T. reesei* for 14 days decreased concentrations of NDF and ADF by 14.4 and 10.0%, respectively. Furthermore, NDFD was increased when brown-rot fungi-treated bean straw was fed to sheep (El-Banna et al., 2010). However, Nurjana et al. (2016) reported that a different *T. reesei* strain, QM6a, decreased NDF and ADF concentrations of Napier grass but did not affect NDFD.

Lack of *in vitro* and *in vivo* studies to examine digestibility and animal performance-enhancing effects of brown-rot fungi are attributable to the long pretreatment time, the need for aerobic conditions for the treatment, and the fact that certain strains of brown-rot fungi degrade desirable polysaccharides, which could reduce the residual nutrient content of treated forages. More research is needed to identify strains that remove or modify lignin in ways that increase accessibility to cellulose and hemicellulose, without degrading these beneficial polysaccharides.

## Summary



Using brown midrib hybrids has been among the most consistent, cost effective and adopted strategies to increase forage fiber digestion and milk production by dairy cows. In this context, more research is needed to examine and validate the efficacy and cost effectiveness of other genetic technologies like low-lignin alfalfa or grasses, seedling-ferulate ester mutants, and transgenic fibrolytic-enzyme secreting forages. Mechanical treatment methods that reduce forage particle size vary in effects on fiber digestibility depending on the particle size achieved. A balance between maintaining physical effectiveness of the fiber and reducing the particle size is critical for such approaches even when they increase intake and facilitate handling and transport of feeds. Chemical treatment methods of improving fiber digestibility are consistently effective, but their widespread adoption has been limited by their caustic nature and high costs. Among the biological treatment techniques, some (yeast products, enzymes and inoculants) have increased fiber digestion and milk production by dairy cows in recent meta-analyses though responses in individual studies have varied. Omic technologies should be exploited to make such products more potent and consistently effective. Other biological treatments (brown and white rot fungi) have considerable potential to improve fiber utilization provided strains used avoid or minimize carbohydrate degradation. Combination treatments like Ammonia-Fiber Expansion or steam-pressure-thermal treatment can reduce the integrity of fiber and increase the digestibility but they are not feasible on farms, as they occur in reactors. More studies on the cost effectiveness of feeding the products are needed as well as studies on adapting the technology for on-farm use.

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# 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, phenylacetyl glycine, 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.Hungate1000.org.nz](http://www.Hungate1000.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<sub>2</sub> and CO<sub>2</sub>. 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<sub>2</sub> (hydrogenotrophic pathway). Hydrogenotrophic methanogens include the genus *Methanobrevibacter*, which is subdivided into the SMT clade (*M. smithii*, *M. millerae*, *M. gottschalkii*, *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<sub>2</sub>-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 defaunation 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 matter 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 auxiliary 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<sub>2</sub>, of global warming. Methane is more potent than CO<sub>2</sub> 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 et 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<sub>2</sub> 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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