

# Proceedings

## 2017 Cornell Nutrition Conference for Feed Manufacturers

79<sup>th</sup> Meeting

October 17 – 19, 2017

Doubletree Hotel

East Syracuse, New York

Cornell University Department of Animal Science

at the

New York State College of Agriculture and Life Sciences

Ithaca, New York



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Dr. Trevor DeVries, University of Guelph  
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Mr. Rodrigo Molano, Cornell University (Ph.D student)  
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# 2017 Conference Program

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## Tuesday, October 17, 2017

### ***Pre-Conference Symposium Sponsored By Phibro Animal Health “The Broad Impacts of the Immune System on Dairy Cattle Production”***

- 1:00 PM Welcome
- 1:10 PM Immunity in Dairy Cattle: What’s A Healthy Cow?  
*Dr. Derek McLean, Phibro Animal Health*
- 1:35 PM Immunity, Inflammation and the Transition Cow  
*Dr. Barry Bradford, Kansas State University*
- 2:20 PM The Direct and Indirect Effects of the Immune System on Reproduction in Dairy Cattle  
*Dr. Peter Hansen, University of Florida*
- 3:00 PM Break
- 3:30 PM Immunity and Ruminant Acidosis/Laminitis  
*Dr. Tanya Gressley, University of Delaware*
- 4:15 PM Immunity and Heat Stress in Dry and Lactating Cows  
*Dr. Geoff Dahl, University of Florida and Dr. Robert Collier, University of Arizona*
- 4:55 PM Wrap-up  
*Dr. Jim Chapman, Phibro Animal Health*
- 5:00 PM Social Hour

## Wednesday, October 18, 2017

- 6:30 AM ***Breakfast, sponsored by Marsyt***
- 7:00 AM Marsyt sponsored presentation  
The Science Behind Natural Alternatives in Production Agriculture  
*Dr. Milena Sevastiyanova, Innovad*
- 8:15 AM Welcome
- 8:20 AM Management of Fresh Cows for Best Behavior  
*Dr. Trevor DeVries, University of Guelph*
- 9:00 AM Can Genomics of Dry Matter Intake in Transition Cows Improve Health and Fertility?  
*Dr. Ron Butler, Cornell University*
- 9:40 AM Presentation of Maynard Award  
*Dr. Tom Overton, Cornell University*
- 9:50 AM **Break**
- 10:20 AM Fatty Acid Nutrition of the Fresh Cow  
*Dr. Adam Lock, Michigan State University*
- 11:00 AM Developing Practical Approaches to Modify Hepatic Fatty Acid Processing and Lipid Mediator Biosynthesis in Dairy Cattle: The Emerging Role of Lipidomics

- Dr. Joe McFadden, Cornell University*
- 11:40 AM Updating the Amino Acid Content of Microbes, Milk, Feed, and Tissue for Application in the CNCPS  
*Dr. Mike Van Amburgh, Cornell University*
- 12:10 PM **Lunch**  
*Mini-Symposium*  
*Disrupters to Animal Source Protein Production as We Know It Today*
- 1:30 PM Lab-Based Meat Production – Science Fiction or Reality?  
*Dr. Mark Post, MD, PhD, Maastricht University*
- 2:20 PM Milk Avoidance and Beverage Alternatives  
*Dr. Dennis Savaiano, Purdue University*
- 3:10 PM Moderated Discussion - Potential Disruptors to the Dairy and Beef Industry - What Does This Mean for Animal Production Systems?  
*Moderator: Dr. Mike Van Amburgh, Cornell University*  
*Panelists: Dr. Mark Post, MD, PhD, Maastricht University and Dr. Dennis Savaiano, Purdue University*
- 3:30 PM **Break**
- 4:00 PM Infrared Milk Fatty Acid Analysis: Experience in the Field  
*Dr. Dave Barbano, Cornell University*
- 4:40 PM The Cornell Milking Sheep Project  
*Ms. Nikola Kochendoerfer (MS student) and Dr. Mike Thonney*
- 5:00 PM Graduate Student Poster Session
- 5:15 PM **Stations Dinner Reception**

## **Thursday, October 19, 2017**

- 6:30 AM **Breakfast sponsored by CHR Hansen**
- 6:45 AM CHR Hansen sponsored presentation  
Moving Towards the New Food World - What Do All These Food Trends Really Mean for the Dairy Producer?  
*Dr. Kelli Hayes, Chr Hansen A/S*
- 8:05 AM Welcome
- 8:10 AM Making Decisions About New Technologies on the Dairy  
*Dr. Tom Overton, Cornell University*
- 8:45 AM Phytochemicals in Microalgae and Benefits in Heat Stress  
*Dr. Xingen Lei, Cornell University*
- 9:20 AM Charlie Sniffen Graduate Research Presentation, sponsored by Kemin Animal Nutrition and Health  
Effect of Dietary Supplementation of Two Forms of a B-Vitamin and Choline Blend on the Performance of Holstein Calves during the Transition and Early Post-Weaning Period  
*Rodrigo Molano (PhD student), Cornell University*
- 9:50 AM **Break**

- 10:20 AM Cropping Considerations for Herds Considering non-GMO Production  
*Mr. Joe Lawrence, Cornell CALS PRO-DAIRY*
- 10:50 AM Ration Considerations for Herds Considering non-GMO Production  
*Dr. Larry Chase, Cornell University*
- 11:20 AM Responses to Methionine Supplementation During the Pre-Fresh Period and Early Lactation  
*Dr. Matias Stangaferro (PhD student) and Dr. Julio Giordano, Cornell University*
- 11:50 PM Feeding the Fresh Cow – Fiber Considerations  
*Ms. Sarah LaCount (PhD candidate), Cornell University*
- 12:20 PM Adjourn

***Post-Conference Symposium sponsored by Mycogen Nutrition Services  
“Bringing It Together: Advancements in Forage Digestibility”***

- 12:20 PM Post-Conference Symposium Lunch**
- 1:00 PM Fiber is Complicated! Understanding aNDF, aNDFom, NDFD, uNDFom in Your Forage Analysis Report and Its Application  
*Dr. Tom Tylutki, AMTS LLC*
- 1:55 PM Moving Forward with Silage Hybrid Evaluation  
*Dr. Greg Roth, Penn State University*
- 2:40 PM It’s the Combination- Scientific Data Review of the First Corn Silage to Bring Together Fiber and Starch Digestibility  
*Dr. Rick Grant, Miner Institute*
- 3:15 PM Adjourn

Proceedings are available for download at <http://ansci.cals.cornell.edu/CNC/>.



## Speaker Biographies

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### **Dr. Dave Barbano**

*Cornell University*

Dr. Dave Barbano is Professor of Food Science at Cornell University. Dave conducts an applied and basic research program on 1) natural cheeses and whey products, 2) improvement of methods of analysis of dairy foods, 3) raw milk and dairy food quality, 4) membrane filtration of milk and whey for protein separation and microbial removal. Recently, Dave has focused on developing new milk analysis measures of cow metabolic health for dairy herd management. Dave also delivers a technology transfer program to communicate research results to the dairy industry and teaches a dairy chemistry course. 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 serves as Director of the Northeast Dairy Foods Research Center program that is funded jointly by national and regional milk promotion units, suppliers, and dairy product manufacturers.

### **Dr. Ron Butler**

*Cornell University*

W.R. (Ron) Butler is a Professor in the Department of Animal Science at Cornell University. He received a B.S. degree in Dairy Science and M.S. in Reproductive Physiology from The Ohio State University and a PhD in Reproductive Physiology from Purdue University. His research and teaching career (42 years) has been directed at regulation of ovarian follicle development, ovulation, and fertility in dairy cattle and other species. Nutrition and reproduction are interrelated and, in particular, negative energy balance in postpartum dairy cows affects metabolic hormones and gonadotropin secretion that determine subsequent health, milk production, and reproductive performance. His current research explores molecular, genetic and hormonal mechanisms in ovarian and liver tissues as most affected by changes in metabolic activity during the transition period and early lactation. The overall goal is to combine dietary strategies and genomic information to enhance reproductive efficiency, health, and profitability of dairy cattle.

### **Dr. Larry Chase**

*Cornell University*

Dr. Larry Chase is a Professor Emeritus of Dairy Nutrition in the Department of Animal Science at Cornell University. Larry served as the General Chairman of the Cornell Nutrition Conference for 30 years. His current research activities are in the environmental impacts of dairy cattle rations and greenhouse gas emissions.

### **Dr. Trevor DeVries**

*University of Guelph*

Dr. Trevor DeVries is a Canada Research Chair in Dairy Cattle Behavior and Welfare and an Associate Professor in the Department of Animal Biosciences at the University of Guelph. Trevor received his B.Sc. in Agriculture from The University of British Columbia (UBC) in 2001. Immediately following he began graduate studies at UBC, focusing his research on dairy cow feeding behavior. After receiving his Ph.D. in 2006, he worked for one year as a post-doctoral researcher at Agriculture and Agri-Food Canada, focusing his research on ruminant nutrition. In 2007 he was appointed as faculty with the University of Guelph. In his current position Trevor is involved in research and teaching in the areas of dairy cattle nutrition, management, behavior, and welfare.

**Dr. Julio Giordano**

*Cornell University*

Dr. Julio Giordano is Assistant Professor of Dairy Cattle Biology and Management in the Department of Animal Science at Cornell University. His expertise is in dairy cattle reproduction, health, and the implications of herd performance on the economics of dairy farms. His basic research focuses on the elucidation of physiological mechanisms controlling reproductive function and changes in physiological parameters during disease in dairy cattle. His applied program incorporates novel technologies to develop new and simplify established reproductive and health management programs for dairy cattle. Through the integration of these basic and applied research components, Dr. Giordano's laboratory strives to enhance the reproductive performance, health, and productivity of cows thus, the economic viability of dairy farms.

**Ms. Nikola Kochendoerfer**

*Cornell University (MS student)*

Niko received her undergraduate degree in Animal Science and Agricultural Management at Anhalt University in Bernburg, Germany in 2012. She has experience managing large-scale sheep flocks in Germany and worked as whole farm consultant in the dairy industry. Currently she's a master's student with Mike Thonney in the Cornell Graduate Field of Animal Science working on nutrition and management of milking sheep.

**Ms. Sarah LaCount**

*Cornell University (PhD candidate)*

Sarah LaCount is a 4th year PhD student in Tom Overton's lab at Cornell University studying the impacts of starch and fiber in transition cow rations. Sarah received her BS in Dairy Science from Virginia Tech in 2013 and completed a 1 year research internship at the William H. Miner Institute before coming to Cornell.

**Mr. Joe Lawrence**

*Cornell CALS PRO-DAIRY*

Joe Lawrence joined the Cornell CALS PRO-DAIRY team in 2016 as a Forage Systems Specialist. He holds undergraduate degrees in engineering and agronomy and completed a master's degree in crop and soil sciences with the Nutrient Management Spear Program at Cornell University. He has worked in the New York dairy industry for over 10 years with a strong focus on a whole farm approach to forage management.

**Dr. Xingen Lei**

*Cornell University*

Xingen Lei received his B.S. and M.S. in China, and Ph.D. from Michigan State University. Since joining the Cornell faculty, he has pioneered in developing a new generation of bacterial phytases and demonstrating their nutritional and environmental values. Lei also pioneered nutritional genomics of selenium in pigs, chicks, and rodents and revealed dual roles of selenium in oxidative stress and diabetes. In 2017, he has received the FASS-AFIA New Frontiers in Animal Nutrition Award and the ASAS Gary Cromwell Award in Mineral Research.

**Dr. Adam Lock**

*Michigan State University*

Dr. Adam Lock is an associate professor in the Department of Animal Science at Michigan State University. Originally from a dairy farm in the southwest of the United Kingdom, Dr. Lock received his PhD from the University of Nottingham and completed a post-doc at that institution as well as at Cornell University. He had a research and teaching appointment at the University of Vermont from 2006 to 2009 before moving to his current research and extension appointment

at Michigan State University in the fall of 2009. Dr. Lock has developed his expertise in ruminant nutrition and physiology. His research and extension programs focus on both dairy production and human nutrition and health, and the interface between these two disciplines. The central theme is fatty acid digestion and metabolism in the dairy cow and the impact of bioactive fatty acids on animal production and human health. Current efforts concern the effect of diet on the production of biohydrogenation intermediates in the rumen, dietary strategies for maximizing milk fat synthesis, applying this knowledge to improve our ability to troubleshoot on farm issues related to milk fat depression, fatty acid absorption in the small intestine, fat supplementation opportunities, and the potential for omega-3 fatty acids to promote dairy cattle metabolism and health. The impact of milk and dairy products on human health, in particular the role of milk fat is also of special interest. He is recognized for his ability to communicate to many sectors, from dairy farmers to dietitians and was awarded the 2011 American Dairy Science Association Young Scientist Award, which recognizes outstanding research by a young dairy scientist during the first 10 years of their professional career.

### **Dr. Joe McFadden**

*Cornell University*

Dr. Joseph W. McFadden has a scientific interest to define the mechanisms of insulin resistance and fatty liver disease in dairy cattle. In 2003, he received a B.S. degree with Distinction in Research from the Department of Animal Science at Cornell University. He then completed an M.S. degree in Animal Science from the University of Illinois with a dairy cattle nutrition focus. In 2009, Dr. McFadden obtained a Ph.D. degree in Dairy Science from Virginia Tech. Following his Ph.D. training, Dr. McFadden gained experience in the field of mass spectrometry-based lipidomics as a postdoctoral fellow in the Department of Neuroscience and the Center for Metabolism and Obesity Research at Johns Hopkins University School of Medicine. In 2012, Dr. McFadden joined the faculty in the Division of Animal and Nutritional Sciences at West Virginia University as an assistant professor of biochemistry where he integrated hypothesis-driven lipidomics within the dairy sciences. Dr. McFadden recently joined the Cornell University Department of Animal Science as the Northeast Agribusiness and Feed Alliance Faculty Fellow in Dairy Cattle Biology. With federal and industry support, Dr. McFadden continues to employ lipidomics as a means to develop practical applications to improve hepatic health and lactation performance in cows.

### **Mr. Rodrigo Molano**

*Cornell University (PhD student)*

Rodrigo was born in Bogota, Colombia, where he also got his Animal Science Degree. He worked in the industry for a couple of years before joining Dr. Mike Van Amburgh's group in 2014, where he has been investigating in the area of calf and heifer nutrition.

### **Dr. Tom Overton**

*Cornell University*

Thomas R. Overton, Ph.D., is Professor of Dairy Management in the Department of Animal Science at Cornell University. Tom is recognized nationally and internationally for his research and extension efforts relating to metabolism, immune function, and nutritional physiology of the transition cow and his work on milk component production in cows. He serves as Director of the PRO-DAIRY program at Cornell, and as Associate Director of Cornell Cooperative Extension works with statewide and regional extension teams within New York to enhance the dairy and agricultural industries in New York State. He teaches the applied dairy cattle nutrition course for undergraduates and co-teaches a course in dairy nutrition for veterinary students.

Tom is a native of northern New York who grew up primarily in Massachusetts. He has a B.S. degree from Cornell University and M.S. and Ph.D. degrees from the University of Illinois. He returned to Cornell as an Assistant Professor in 1998 and was promoted to Associate Professor in 2004 and to Professor in 2013. He has authored or coauthored more than 80 peer-reviewed scientific publications and numerous technical articles for conference proceedings, extension publications, and popular press articles. He was awarded the Cargill Animal Nutrition Young Scientist Award by the American Dairy Science Association in 2006 and the ADSA Foundation Scholar Award in 2007. In 2013, he was named a Faculty Fellow of the David R. Atkinson Center for a Sustainable Future at Cornell University.

**Dr. Mark Post**

*Maastricht University*

Professor Mark Post is an MD, PhD and chair of the Physiology Department at Maastricht University. His main research interest is the engineering of tissues for medical applications and for food, which has led to the development of cultured beef from bovine skeletal muscle stem cells in an effort to supplement and transform the traditional meat production through livestock. In August 2013, he presented the world's first hamburger from cultured beef. He is CSO and co-founder of two companies, MosaMeat and Qorium that will commercialize cultured meat and cultured leather.

**Dr. Dennis Savaiano**

*Purdue University*

Dennis Savaiano is Director of the North Central Nutrition Education Center of Excellence, a USDA NIFA-funded center. He is also the Associate Director and Purdue liaison for the Indiana Clinical and Translational Sciences Institute Community Health Engagement Program. He has published on school community interventions focused on improving the diets of youth (particularly young women) and has led/developed many of the successful Purdue Extension efforts focused around health as dean of Purdue's School of Consumer and Family Sciences for 15 years. Professor Savaiano has studied lactose digestion for the past 30 years. His research group has studied numerous factors which influence lactose digestion and tolerance including lactose load, gastric and intestinal transit, the use of lactose digestive aids, colon fermentation of lactose and the consumption of fermented dairy foods and lactic acid bacteria. Work in his lab is currently aimed at understanding why these maldigesters have developed a strong belief that is not supported by blinded, clinical trials. Further, he is interested in methods of intervention that will allow "lactose intolerant" individuals to learn that they can consume dairy foods without experiencing gastrointestinal symptoms. He continues to assess lactose digestion and tolerance in special populations such as adolescent women. Finally, he is working with the dairy industry to attempt to develop food products that are well tolerated by the lactose maldigester.

**Dr. Matias Stangaferro**

*Cornell University (PhD candidate)*

Matias Stangaferro is a PhD candidate working in the Dairy Cattle Biology and Management Laboratory in the Department of Animal Science at Cornell University. Matias holds DVM and MS degrees from the Universidad Nacional Litoral in Argentina. Before joining Cornell as a Fulbright grantee, Matias was a Senior Lecturer of Theriogenology in the Veterinary College at Universidad Nacional Litoral. Currently, Matias' research focuses on the design and implementation of management strategies to improve dairy cattle reproduction, nutrition, and health.

**Dr. Mike Thonney**

*Cornell University*

Mike Thonney received his undergraduate degree in Animal Science at Washington State University in 1971, followed by MS and PhD degrees at the University of Minnesota before joining the Department of Animal Science at Cornell in 1975. He currently teaches about sheep, beef cattle, and meat. His research background includes projects on growth and development of cattle and sheep, effect of candidate genes on muscle growth and aseasonal breeding in sheep, and vaccination and management of sheep. Currently, he is working with Niko Kochendoerfer on feeding and managing sheep in short, frequent lactations.

**Dr. Mike Van Amburgh**

*Cornell University*

Mike Van Amburgh is a Professor in the Department of Animal Science and a Stephen H. Weiss Presidential Fellow at Cornell University where he has a dual appointment in teaching and research. His undergraduate degree is from The Ohio State University and his Ph.D. is from Cornell University. He teaches multiple courses and leads the Cornell Dairy Fellows Program, advises approximately 50 undergraduate students and is the advisor for the Cornell University Dairy Science Club.

For the last 20 years, a major focus of his research program has been to describe the nutrient requirements of dairy calves and heifers and aspects of endocrine control of developmental functions such as mammary development. This has evolved into describing and working to understand factors in neonatal life that establish lifetime productivity functions and outcomes. Mike currently leads the development of the Cornell Net Carbohydrate and Protein System, a nutrition evaluation and formulation model used worldwide and through that effort is focused on enhancing the efficiency of nutrient use by ruminants to improve the environmental impact of animal food production. A significant focus of his current work is to understand whole animal and ruminal nitrogen metabolism and amino acid supply and requirements to enhance the development of the Cornell Net Carbohydrate and Protein System. Further, his group is active in developing methods to better describe the interaction between forage and feed chemistry, rumen function and nutrient supply to compliment the model.

He has authored and co-authored over 70 journal articles and many conference proceedings and is the recipient of several awards including the American Dairy Science Foundation Scholar Award, the Land O'Lakes Teaching and Mentoring Award from ADSA, the American Feed Ingredient Association Award for Research, the CALS Professor of Merit Award and the CALS Distinguished Advisor Award.

*PRE-CONFERENCE SPEAKERS*

**Dr. Barry Bradford**

*Kansas State University*

Dr. Bradford is a Professor of Metabolic Physiology in the Department of Animal Sciences and Industry at Kansas State University. Raised on a cow/calf operation, he now studies dairy cattle with a focus on nutrition/immunity interactions, signaling effects of nutrients, and utilization of low-input feedstuffs. In addition, he teaches more than 180 students per year in animal nutrition and physiology courses.

**Dr. Bob Collier**

*University of Arizona*

Dr. Collier received his B.S. and M.S. degrees in Zoology from Eastern Illinois University and his Ph.D. in Dairy Science from the University of Illinois. Previously with the University of Florida and Monsanto Company, he is currently Emeritus Professor at the University of Arizona. His areas of expertise include environmental and lactation physiology, endocrinology and molecular biology.

**Dr. Geoff Dahl**

*University of Florida*

Dr. Dahl is Professor & Chair of the Department of Animal Sciences at the University of Florida. His research and Extension program focuses on the effects of environmental factors, especially heat stress and photoperiod, during the dry period of dairy cows and their calves.

**Dr. Tanya Gressley**

*University of Delaware*

Dr. Gressley is an Associate Professor in the Department of Animal and Food Sciences at the University of Delaware. She received her B.S. and M.S. degrees in Animal Science from the University of Maryland, and Ph.D. Dairy Science from the University of Wisconsin. Her research program focuses on dairy cattle nutrition and the impact nutrition has on cow health.

**Dr. Peter Hansen**

*University of Florida*

Dr. Hansen is a Distinguished Professor in the Department of Animal Sciences at the University of Florida. He received his B.S. in Agriculture from the University of Illinois, and M.S. and Ph.D. in Endocrinology-Reproductive Physiology from the University of Wisconsin. His overall research goal is to understand determinants of pregnancy success in ruminants. This includes research on molecules regulating programming of the embryo before implantation; identification of genes controlling fertility; and the effects of cellular stress on the embryo. These studies of embryo physiology have led to new management strategies to improve fertility in dairy cows.

**Dr. Derek McLean**

*Phibro Animal Health*

Dr. McLean has worked in the area of livestock animal health for approximately 20 years including research with dairy, beef, poultry and swine. He earned a Ph.D. in Animal Science from Oregon State University followed by research and academic positions at Northwestern University and Washington State University. Derek has been working with the Phibro Animal Research team for five years.

*POST-CONFERENCE SPEAKERS*

**Dr. Rick Grant**

*Miner Institute*

Rick Grant was raised on a dairy farm in northern New York State. He received a B.S. in Animal Science from Cornell University, a Ph.D. 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. Greg Roth**

*Penn State University*

Dr. Greg Roth is a Professor in the Department of Plant Science at Penn State and has an extension/research appointment focusing on grain crop management. He has been a member of the faculty at Penn State since 1989. He has a B.S. and Ph.D. in Agronomy from Penn State University and a M.S. from Virginia Tech. In his position as the extension grain crops specialist, he has provided leadership for the Corn Silage Hybrid Evaluation program since 2000. His work has also included various corn silage management topics, alternative forages such as small grains and sorghum, and cover cropping.

**Dr. Tom Tylutki**

*AMTS LLC*

Tom Tylutki is President and CEO of AMTS, Agricultural Modeling and Training Systems. He holds a PhD from Cornell University and has been involved in the development and training of the original Cornell model since 1990.

*BREAKFAST SPEAKERS*

**Dr. Kelli Hayes**

*Chr Hansen A/S*

Based in Copenhagen, Denmark, Kelli Hayes is Director, Corporate Strategy & Business Development at Chr Hansen, where she leads projects of strategic importance to the global organization. Her previous experience includes working for McKinsey & Company's South African office as well as Wells Fargo's Corporate Banking division in both San Francisco and New York City. She holds a PhD in Moral Philosophy and an MPhil in Applied Ethics from the University of Stellenbosch in South Africa as well as a Bachelor in Business Administration from the University of Texas at Austin.

**Dr. Milena Sevastiyanova**

*Innovad*

Dr Milena Sevastiyanova (Technical & Commercial Manager – INNOVAD) received her master degree in veterinary medicine in 1996. Then she worked 15 years related with pharmaceuticals - Merial S.A.S as a Veterinary Professional Service Provider and Swine Technical & Marketing Support. Dr Sevastiyanova has accumulated extensive experience with swine production while directly involved in swine production management with BONI Holding in Bulgaria. Now with INNOVAD, Dr Sevastiyanova is supporting our international partners with her technical service experiences.

## **IMMUNITY IN DAIRY CATTLE: WHAT'S A HEALTHY COW?**

D. McLean  
Phibro Animal Health Corporation

The maintenance of health for a dairy cow requires the interaction of multiple physiological systems during dynamic shifts in tissue and organ function. Stable immune function during these shifts provides the animal the foundation for health and optimal performance. Health and immune function can be impacted by physiological strain induced from many factors including environment (heat), pathogen challenge and nutritional imbalance. The reaction in the animal is either induction or repression of immune function, both of which can cause inflammatory cascades that can compromise health and productivity. The dairy cow possesses several unique characteristics that make it an ideal livestock species for analysis of the regulation of immune function. Dairy cows have distinct developmental, management and production cycles that last weeks to months and biological samples can be collected repeatedly during these cycles. In addition, dairy cows are exposed to metabolic, environmental and nutritional challenges induced by calving, lactation and housing. Balance of these challenges to maintain health is key to productivity. Measurements that provide a real-time index of health or possibly a predictive index of health would be of great value for all levels of dairy production. The ability to strive for optimal dairy cow production and health requires a repeatable, nondirected analytical approach to identify pathways and markers of known importance in cow immune function as well as shifts in the relative abundance of these markers. This approach may provide an analytical definition of a “healthy cow”. Progress is being made in this direction, but a thorough understanding of the inputs needed for the optimal productive and healthy cow is ongoing.

The maintenance of animal health was unlikely a major consideration when the domestication of cattle originated 8,000 to 10,000 years ago (Bollongino et al., 2012). However, the robust nature of aurochs and the ability of these animals to thrive in conditions that were not optimal for human survival was a major driver of domestication. The individuals participating in this key step in the development of modern agriculture initiated a sequence of events that has continued for thousands of years. This achievement demonstrates two important aspects of cattle. First, the plasticity of the animal to acclimate to various conditions. Second, the long-term interest of humans to develop value added products with specific characteristics.

### **SELECTION OF PRODUCTION TRAITS**

The ability to facilitate the development of organisms for economically important traits through genetic selection is a hallmark of modern agriculture. The modern dairy industry has been shaped with the use of identification of desirable genetic traits and utilization of artificial insemination to facilitate selection and propagation of the traits. With production goals in mind, fewer than 10 of the 800 breeds of cattle are used today for large scale milk production. Even within this small group of dairy focused breeds,



differences for traits important for the dairy industry have been used to advantage by breed associations for marketing or formulas for profitability. These differences may seem minor to outside observers but represent unique qualities that can shift profitability in relatively short timeframes (Liu et al., 2010). Similarly, the physical appearance of modern dairy cattle has changed little over the past several hundred years. The major changes that resulted in the high production of the modern dairy cow are therefore, largely internal and biological, based on how the physiological systems can tolerate the demands to accomplish maintenance and production through homeostatic regulation.

The shift to high dairy cow productivity is an excellent example how the merger of multiple technologies resulted in major progress in productivity (Akers, 2000). At the farm level, optimized cow housing and disease control, technology advances in milking equipment and computerization of record keeping and for accurate and precise ration formulization improve general management. Quantitative genetics and genomics are used for selection before cows complete their production lifespan, taking advantage of real-time collection of milking data from computerized farm systems for accelerated selection and data sharing (Powell and Norman, 2006; Windig et al., 2006). The use of reproductive technology for artificial insemination and embryo transfer for the rapid expansion of animals with desirable genetics has provided the means to support efficient expansion of herds selected for milk production. Further advancement of cow productivity is expected with continued use of new technology and knowledge (Chesnais et al., 2016). The challenge with emphasis on production, in this case milk, is to avoid negative consequences to other physiological mechanisms that are important for the animal to maintain homeostasis (Rauw et al., 1998).

As stated earlier, while physically similar, the production level of the modern dairy cow is unrecognizable today from even 50-100 years ago. The internal changes have been linked to improved feed efficiency and less energy going to physiological maintenance (VandeHaar et al., 2016). This is a remarkable change that suggests the question: what is lost? Unfavorable genetic correlations have been reported between milk production and reproductive function, metabolic disease during transition, mastitis, displaced abomasum, dystocia and mobility (Rauw et al., 1998; Oltenacu and Broom, 2010; Adamec et al., 2006). The most apparent aspect of these unfavorable side effects of selection for increased production is the negative economic impact for producers (Parker Gaddis et al., 2014). The link between increased milk performance and negative impact on other physiological systems is generally linked to impaired metabolic balance due to the increased need for energy for milk production following calving. Based on this concept, the ability to nutritionally manage and select cows to reduce negative energy balance to prevent increased disease may be possible (Gervais et al., 2017).

The clear and understandable decision is to select cows for milk production. There are consequences to this decision including possible increases in disease or limitations on physiological systems that will negatively impact cow health and ultimately profitability. A producer is faced with the question of how can we maintain productivity and cow health to maximize production? Can we select or balance the selection of traits that optimize production, feed efficiency and cow health so that profitability can still be achieved?

Measurement of milk production is straightforward. Although more complicated than milk production, feed efficiency is measurable and selection for these traits is an active area of research (Pryce et al., 2014; VandeHaar et al., 2016; Seabury et al., 2017). The measurement of cow health is another level of complexity beyond milk production and feed efficiency. When discussing cow health, the first question may be: what should be measured? Then other questions follow – when in the life or production cycle of a cow should health be measured? How frequently should health be measured? And, what does the measurement mean?

## COW HEALTH ASSESSMENT

The establishment of dairy cow health starts when it is extremely difficult to collect a sample. Maternal fetal interactions *in utero* due to the environmental conditions and nutritional maintenance are known to impact calf health, development and production potential for the offspring as an adult (Guo et al., 2016; Batistel et al., 2017). Following birth, effective management of the nutrition and environment for calves is critical for development and growth for a productive lifespan (Bach, 2012; Langel et al., 2015). While these are interesting and worthwhile topics, the focus of this paper is to discuss how we can evaluate the health of adult cows.

A simple index for health measurement for humans include body mass index, biological age, waist measurement, blood pressure and heart rate. Unfortunately for cows, these measurements, although useful in general, do not provide enough information for the complex physiological challenges that a growing calf, heifer, dry and lactating cow are experiencing. However, many measurements that are a routine part of the daily management of cows such as feed intake, milk production and movement are a straightforward indication of health status and can be obtained from records (LeBlanc, 2010). These records and producer-generated health records may be an unrealized resource for genetic selection (Parker Gaddis et al., 2014). However, the accuracy and completeness of the records may be a limitation to this approach. The next level of analysis of health status is more invasive than routine observation or records review. This step is the beginning of moving from simple visual and record observation to a potential Schrodinger's Cat model of cow health status and evaluation (Kovac, 2016). More specifically, the cow is healthy until you open the cow box and begin measuring blood components or milk or other biological samples. At that point, the amount of information gained can be almost endless and using the information to gain knowledge becomes challenging.

There are, of course, many measurements that are helpful to evaluate cow health, especially during specific phases of the production cycle. Blood calcium is extremely useful for determining the likelihood of milk fever following calving (Leno et al, 2017). Blood non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB or BHBA) are useful and straightforward assays to determine if a cow is experiencing ketosis and thus increased likelihood for metabolic disease or undernutrition (LeBlanc, 2010; Agenäs et al., 2006). Similarly, the capacity of components of blood to carry oxygen, the blood cell content and amounts and type of proteins in the blood are indicative of a basic health

status. The number of blood cells and the ratios of certain blood cells are used by veterinarians regularly to evaluate the overall health status of a cow. These measurements are valuable when extreme shifts occur and are used to determine treatments. These values also provide a foundation for comparison for research projects focused on manipulation of animal health to gain knowledge for potential treatments or preventative strategies. Now the question becomes are these measurements enough?

The most fundamental physiological activity is metabolism to maintain energy resources for tissue and organ function. The extreme metabolic demands of a cow during its life cycle are well known, but the energy needs at the post-calving interval and the connection of metabolic disease and immunosuppression do not require a great intellectual leap to understand why cows are susceptible to health issues. To understand how a cow stays healthy, the balance of production and health need to be understood through the connection of metabolism and immune system function. The immune system is a complex tissue, organ and cellular organization that requires coordination to prevent inappropriate activation. This coordination is primarily through the release of molecules such as cytokines that activate or suppress immune system function. The activation is key to prevent pathogen infection that can suppress health, but the cost can be significant through energy use, fever, inflammation and tissue damage. When considering the metabolic drain of immune activation during a health challenge, the physiological systems must make a resource allocation decision and tolerate the change. This resource allocation shift away from other systems to immune function is likely due to the immediate requirement for response to a health challenge. Indeed, circulating immune cells primarily rely upon glucose and fatty acids for energy (Wolowczuk et al., 2008). This demand has been investigated at both the animal level and the cell level.

## ENERGY DEMANDS OF IMMUNE SYSTEM

The elevation of body temperature above the normal range, also known as fever, is an immune system regulated response to gain an advantage over an infectious agent. The physiological mechanism to induce a fever is primarily mediated by the hypothalamus that includes cells with receptors to detect pyrogens circulating in the blood. Pyrogens can be externally derived such as lipopolysaccharide (LPS) or internally produced molecules including interleukin (IL) 1 alpha and beta (IL-1  $\alpha$  and IL-1 $\beta$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) secreted by activated immune cells (Steffler et al., 1996). The response is energetically demanding including increased skeletal muscle metabolism and peripheral vasoconstriction. Depending on the species, a fever requires a 7-15% increase in caloric energy production to increase the body temperature one degree Celsius (Elia, 1992; Demas et al., 1997). Extending the analysis to include all energetic demands of an experimentally induced infection, it has been determined in birds that infection will increase metabolism 7-29% above pre-infection measurements (Nilsson, 2003; Martin et al., 2003). These increases exert a significant strain on an animal operating at a baseline, but in the case of a lactating cow with the additional requirements of milk production and potential support of a fetus, the immunological and resulting energetic strain would be expected to have a long-term negative health impact. To specifically investigate the energetic impact of an activated immune system a technique

was developed to estimate the use of glucose in lactating cows following artificially induced activation of the immune system (Kvidera et al., 2016). The researchers determined that the activated immune system in a lactating cow uses approximately 1kg of glucose (Kvidera et al., 2017). This significant glucose drain will result in a negative impact for production and health.

Detailed investigation of how specific cells of the immune system have increased energetic requirements upon activation adds more to the understanding of why so much energy is required during activation. Generating an effective immune response requires cellular activation of proliferation, generation and secretion of autocrine and paracrine factors and potentially active cell movement, all activities that require enhanced energy. Immune cells, circulating and those residing in tissues, are generally considered to maintain a low basal activity that is regulated by cytokines. While increased energy requirements are universal for activated immune cells, the functional needs of cells differ. For example, lymphocytes must present antigens, secrete immunoglobulins and cytokines, and proliferate (Sordillo, 2016; Hume et al., 1978). This energetic demand is mostly met through glucose while it must be emphasized the major checkpoint for limiting lymphocyte metabolism is the availability of trophic signals, not the availability of glucose (Buttgereit et al., 2000).

Components of the innate immune system include epithelial barriers, non-cellular factors such as complement, endothelial cells and immune cells including neutrophils, macrophages, dendritic cells and natural killer cells. Basal metabolism regulates epithelial cell barriers but upon exposure to pathogens, cells that comprise these regions will initiate interaction with resident immune cells requiring increased energy requirements. The gut epithelium may be important for local regulation of the gut immune system by maintaining the balance of nonpathogenic bacteria to prevent immune system activation. A rapid shift in gut epithelial signaling when the commensal bacteria equilibrium is disrupted may allow passage of pathogenic bacteria through the epithelia barrier to stimulate immune activation while commensal bacteria do not move past the epithelia surface (Wolowczuk et al., 2008). While glucose is the primary energy source for cytokine secretion and antigen presentation, these cells also require lipids for membrane turnover associated with phagocytosis. Likewise, upon activation neutrophils require lipids and energy for phagocytic activity. Despite high turnover and short lifespan, neutrophils are critical for an effective immune system response. Neutrophils are terminally differentiated and thus do not require energy for proliferation but they have few mitochondria and must rely on glucose for most energetic requirements following activation (Maianski et al., 2004). The synthesis of components of the reactive oxygen species (ROS) cascade and secretory activity associated with the release of neutrophil extracellular traps (NETs) are almost exclusively dependent of exogenous glycolysis (Rodriguez-Espinosa et al., 2015).

Based on the importance of glucose and fatty acids for immune cell function, variation in concentrations of these key metabolic molecules affects immune responsiveness. An imbalance of metabolism will disrupt the production of immune system regulatory factors. The connection with metabolic challenges faced by dairy cattle

are therefore direct. The physiological and endocrine changes that occur during calving strain the bovine metabolic system. The periparturient period is characterized by negative energy balance and immunosuppression (Burton and Erskine, 2003; Aleri et al., 2016). Can indices of metabolic function be connected with immune system responsiveness? Measurement of NEFA and BHBA are common during the periparturient period to determine the metabolic strain on the animal. In addition, serum NEFA concentrations above the normal range have been linked to suppression of immune cell function (Ster et al., 2012). Thus, analysis of molecules that indicate metabolic status such as glucose, NEFA and BHBA in circulating blood can be useful for evaluation of metabolic status and potentially the activity of the immune system as an approach to gain information of overall cow health. Should metabolic stability of a cow be used a selection criteria of the ability of the cow to maintain a healthy immune system?

## DEVELOPMENT OF HEALTH INDEX

The link between immune system function has been established in multiple species including research models and humans (Wolowczuk et al., 2008). However, this regulatory system may not be as closely linked in ruminants and especially a high producing dairy cow. The strong selection for production in dairy cows may generate a physiological system balanced for resistance and tolerance. Could the immune system of ruminants, with a unique digestive system compared to other mammals, be less dependent on extracellular glucose or fatty acids and therefore generate a limited picture of immune system health in dairy cows? Limitations associated with the measurement of only metabolites to evaluate metabolic health during the periparturient period have generated investigation into more complex analyses. Metabolic and immunological strain starts during the dry period and continues into early lactation so expansion of the list of metabolites to measure provides the opportunity to develop an index to not only describe animal health but perhaps predict cows that may be high risk to develop disease. In addition, this approach can provide a more advanced understanding of how organ systems are functioning.

Moyes and colleagues (2013) measured urea nitrogen, albumin, cholesterol, NEFA, glucose, and BHBA to create an index for physiological imbalance. This index was compared to calculated energy balance. It is noteworthy that the physiological imbalance index was more effective than other measurements to predict cows during the prepartum period that had a higher risk to develop disease. Physiological imbalance index and NEFA were better indicators of disease during the first week of lactation than other factors measured (Moyes et al., 2013). This approach suggests that predictors for disease risk can be developed, but the complexity of cow physiology during early lactation provides greater challenges. Trevisi and colleagues (Trevisi et al, 2012; Bertoni and Trevisi, 2013) developed a liver functionality index as a tool to characterize the extent of the inflammatory status and health as a predictor for a difficult or smooth transition from gestation to lactation. The index was developed by measuring albumin, cholesterol and bilirubin. Further studies have demonstrated that cows with greater indices of liver function produced more milk (Zhou et al., 2016). The examples suggest that analyzing

multiple metabolites from serum and potentially focusing on specific organ systems, may provide a means to establish a health index for cows.

The connection of immune traits with production, fertility and health would represent a useful approach to combine physiological mechanisms with production. An interesting approach was used to determine if cow immune traits correlated with dairy cow health, reproduction and productivity based on characteristics of immune cells (Banos et al., 2012). Cows from a controlled research herd were sampled over a 10 month period and samples were used to determine if correlations between disease and immune system traits were present. Some interesting data were reported demonstrating that certain immune cells, such as CD335+ natural killer cells were positively correlated with the incidence of mastitis during the week of sampling. Other examples include higher ratios of CD4+:CD8+ cells associated with lower somatic cell counts and a higher percentage of T cells were associated with poorer conversion of feed to milk (Banos et al., 2012). These unique findings suggest that creative investigation can lead to interesting and potentially useful links between immune system traits and production traits. Similarly, a project using this research herd generated additional information regarding production traits and immune cell markers such as relationships between CD4+:CD8+ and live weight (Denholm et al., 2017). Interestingly, they also reported a negative correlation between percentage of eosinophils and milk yield, feed intake, dry matter intake and metabolizable energy intake (Denholm et al., 2017). These reports demonstrate that selection for a combination of immune cell traits and production traits may improve animal health and fitness.

Our current recognition of a healthy cow is based on intake, lactation, metabolic balance, reproductive efficiency and the ability to repeat the production cycle. The dynamic nature of dairy cow life cycle physiology requires a more comprehensive assessment of immune system and animal health due to the numerous challenge a dairy cow will encounter. The investigation of new approaches to measure immune system health and how it is controlled at the physiological and cellular level in dairy cows is an ongoing goal. The current direction is to measure multiple endpoints associated with immune function, metabolism, pyrogens, cell characteristics and organ function to develop indices of health. A single timepoint health index should only be a first step, multiple timepoint assessment and predictive indices need to be a long-term goal. Such a measurement may be useful as a predictor of future health and productivity. This goal may be the most beneficial to connect research with producers. Is “animal health” the first response a producer will state when asked what the primary goal of your operation? Embedded in the question and the goal are a list of factors that can and cannot be controlled. If a measurement or index can be provided, the response “animal health” may become more relevant and provide new directions for nutritional and managements strategies.

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# IMMUNITY, INFLAMMATION, AND THE TRANSITION COW

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## INTRODUCTION

As more products enter the market each year with claims around immune modulation and anti-inflammatory effects, livestock producers and nutritionists are being challenged to become conversant in yet another specialty. This paper will focus on the underlying changes in immune function that are apparent in transition dairy cattle, how inflammatory signaling is involved, and what we know about how and if we should attempt to alter this phenomenon.

### WHY WORRY ABOUT IMMUNITY?

Large-scale analysis of dairy herd records suggests that, around the globe, transition cow problems account for over half of mature animal health problems on a typical dairy farm. There are some obvious risks to cows immediately after calving, including the potential for latent mastitis cases to re-emerge at the onset of lactation and the tissue trauma from calving. However, there is also a well-documented alteration in immune function during the weeks around calving (Kerhli, 2015). In particular, the function of innate immune cells seems to be consistently impaired. Innate immune cells are those involved in quick recognition and clearance of pathogens, independent of pathogen-specific memory (antibodies).

Why is the immune system of transition cows suppressed? The exact reasons for decreased immune function during the transition period are complex. However, studies with mastectomized cows suggest that the primary driver is not gestation and calving, but rather lactation and the metabolic changes that come with it (Nonnecke et al., 2003). Numerous large studies have demonstrated that metabolic diseases (e.g. ketosis) put cows at higher risk of contracting clinical infections; likewise, cows with infectious diseases (e.g. metritis) are also at higher risk of subsequent metabolic disorders. The inter-dependent nature of the immune and metabolic systems in the animal are only now becoming clear, but high blood ketone and non-esterified fatty acid concentrations as well as hypocalcemia are known to limit the responsiveness of immune cells to pathogenic signals. Cows with excessive body condition experience more dramatic drops in immune function at calving, possibly as a consequence of oxidative stress. As a result, nutrition of the transition cow can have a large influence on immunity during this time, even beyond the vitamins and minerals that have received focus in the past.

There is some direct evidence that poor immune responsiveness in the transition period is predictive for incidence of infections during this time. In one study, 5 of 31 cows were identified as poor immune responders 4 weeks before calving. All 5 of these cows

developed clinical infections during the first 2 months of lactation, whereas only 3 of the other 26 cows did so (Catalani et al., 2013). A much larger study of 458 Holstein cows demonstrated that a measure of antibody-mediated immunity was highly predictive of mastitis incidence, with the top quintile showing a 42% lower mastitis incidence than herdmates (Thompson-Crispi et al., 2013). It is likely that the high rate of infections in early lactation can be attributed in part to immunosuppression.

## WHAT DOES INFLAMMATION HAVE TO DO WITH TRANSITION COWS?

Inflammation is a key component of the immune response to infection or tissue damage. Immune cells that first sense pathogens or signs of traumatized cells release signals that activate pain sensors, promote blood flow to the local tissue, and cause fever, accounting for the traditional signs of inflammation. Additionally, the systemic effects of inflammation include an alteration of liver function, typically called the acute phase response. Most of these responses are beneficial for recruiting innate immune cells to the site of immune activation and for inhibition of bacterial growth, but they come at a cost to the animal. Importantly, inflammation can occur in the absence of a true pathogen challenge and can occur without the traditional signs of focal pain, swelling, and redness. When blood markers of inflammation are elevated in the absence of clinical signs, it is often referred to as sub-acute inflammation.

The presence of an acute phase response in postpartum dairy cows is well-established (Bradford et al., 2015). Although early studies focused on associations between inflammatory markers and diseases such as mastitis and metritis, numerous studies in the past decade have demonstrated that inflammatory and acute-phase mediators are elevated in the days after parturition, even in cows that are apparently healthy. This growing body of evidence suggests that either the processes of parturition and galactopoiesis induce inflammation directly or that infections or endotoxin affect far more fresh cows than is currently recognized. Whatever the explanation, the prevalence of post-calving inflammation raises important questions about the implications for early lactation cows.

Although most transition dairy cows apparently experience a period of inflammation, the magnitude of this inflammatory condition varies greatly between cows. Bertoni et al. (2008) assessed the importance of this variation by measuring a panel of inflammatory markers and separating transition cows into quartiles for degree of inflammation. Cows in the highest quartile had significantly lower milk yields than those in the lowest quartile throughout the first month of lactation, differing by 20% on day 28 of lactation (Bertoni et al., 2008). One metric that has been used in this respect is paraoxonase, a plasma biomarker that is potently suppressed by a variety of inflammatory stimuli. Transition cows with high paraoxonase concentrations, in addition to having lower concentrations of acute phase proteins and reactive oxygen metabolites, produced 4,346 lb more milk (24%) over 305 days than those in the lowest quartile for paraoxonase (Bionaz et al., 2007). Other findings suggest that stronger inflammatory responses in the first week of lactation are associated with decreased whole-lactation milk yield (Huzzey et al., 2015). Plasma concentrations of haptoglobin (an acute phase protein) greater than

1.1 g/L were associated with a 2,088 lb decrease in 305-day mature equivalent milk yield, and elevated haptoglobin was also associated with a 19% decreased risk of conception. Abnormally high markers of inflammation are associated with poor production, health, and fertility outcomes.

## IMMUNE PROMOTION TOOLS

With the growing interest in animal characteristics influencing infection risk, a number of factors have emerged as important for supporting strong immunity. Data currently available suggest that cows have improved transition immune function when: 1) they are not exposed to significant heat stress during the dry period (do Amaral et al., 2011); 2) they calve with a BCS between 2.5 and 3.5 (Graugnard et al., 2012; Esposito et al., 2014; Crookenden et al., 2017); 3) they are supplemented with antioxidants during the dry period (Spears and Weiss, 2008); 4) total serum total calcium concentrations are maintained near 9 mg/dL (Martinez et al., 2012; Martinez et al., 2014), and 5) blood BHBA and NEFA concentrations stay below 1 mM during the transition (Grinberg et al., 2008; Contreras and Sordillo, 2011; McArt et al., 2013). Considering the immune system of the transition cow does not necessarily require a change in recommendations for management during this period, but can provide additional motivation to prevent heat stress, provide sufficient access to feed, manage body condition, support calcium homeostasis, and monitor oxidative balance.

Beyond these best practices for transition cow management, a variety of dietary and pharmaceutical products are being marketed for the explicit purpose of improving immune function. Vaccines have obviously been a very useful tool in promotion of adaptive immunity for decades, and the ongoing development of a vaccine against metritis-causing pathogens may soon bring a new weapon to bear on a frustrating problem (Machado et al., 2014). On the other hand, pharmacological tools for promotion of innate immunity have not been available for livestock until very recently. Granulocyte colony-stimulating factor (GCSF) is a signal used by the immune system which has been adapted into an injectable prophylactic treatment used prior to the period of immunosuppression. The GCSF treatment stimulates the development and maturation of neutrophils, resulting in a fairly dramatic increase in the population of these key innate immune cells in circulation. In conditions favorable to environmental mastitis, the administration of GCSF significantly decreases the incidence of clinical mastitis (Hassfurther et al., 2015).

Dietary agents are also being used as immune modulators, although the exact modes of action for these feed additives are more elusive. We recently reported that a dietary yeast product enhanced antibody response to vaccination and stimulated greater gut release of IgA, which is able to bind to and carry pathogens out of the gut (Yuan et al., 2015). A large-scale analysis of commercial farm responses (off-on) to a different feed supplement was presented recently, suggesting beneficial effects on farm-recorded mastitis and mortality (Chapman et al., 2016). Such dietary components can likely alter the responsiveness of the immune system by interacting with immune sentinels lining the

gut and/or by altering gut epithelium function, but other mechanisms cannot yet be ruled out.

## RESPONSES TO ANTI-INFLAMMATORY TREATMENTS

Motivated by evidence linking early lactation inflammation to decreased health and productivity, we conducted a study with 78 cows assigned to either control or sodium salicylate delivered via drinking water (2 g/L) for the first 7 days of lactation (Farney et al., 2013). Sodium salicylate is a member of the non-steroidal anti-inflammatory drug (NSAID) class, and is the parent compound of aspirin. At first the results did not look very promising, with no improvement in metabolic health and no increase in early milk yield. However, as lactation progressed, the oldest cohort of cows treated with salicylate (those in parity 3 and greater) responded by producing 21% more milk over the full lactation, and fully 30% more milk fat, than parity-matched controls. On the other hand, primiparous cows treated with salicylate tended to produce less milk, suggesting a potential parity difference in either baseline inflammatory status or response to inflammatory signals.

We subsequently completed a follow-up study to evaluate whether postpartum treatment of multiparous cows could increase whole-lactation productivity of cows on a commercial farm. To facilitate treatment in a commercial setting, we shortened postpartum treatment to 3 days (sodium salicylate) or 1 day (meloxicam) and compared them to placebo treatments (Carpenter et al., 2016a) across 153 cows. Despite this very limited treatment window, cows treated with either NSAID produced about 10% more milk over the whole lactation compared to placebo. Over the 365 days following treatment, meloxicam also tended to delay removal from the herd based on survival analysis ( $P = 0.06$ ; 30, 35, and 38 of 51 cows remained at 365 d postpartum for control, salicylate, and meloxicam, respectively). Meloxicam primarily affected early-lactation culling, and health records recorded by the farm suggested that metabolic disorders accounted for most of this decrease.

Several other groups in a variety of countries have failed to observe significant impacts of postpartum anti-inflammatory treatment on milk yield, and it remains to be seen whether a treatment paradigm can be found that is consistently effective. However, we believe that impacts on long-term milk yield likely require treatment relatively early after calving (though not before the placenta is cleared); that treatment responsiveness is not limited to cows with calving difficulties; and that milk yield must be monitored for at least 60 days into lactation to have a good chance to observe the impact of anti-inflammatory treatment.

The use of anti-inflammatory drugs to treat nonspecific postpartum inflammation is not currently approved. Therefore, it is worthwhile to consider whether some feed ingredients might offer the same anti-inflammatory benefits without the use of regulated pharmaceuticals.

Polyphenols are a diverse class of compounds found in nearly all plants in varying concentrations. Some polyphenols have been clearly shown to have potent anti-

inflammatory effects, and a recent study demonstrated some exciting responses in dairy cattle during the transition period. Winkler et al. (2015) supplemented cows with a feed supplement containing green tea and curcuma extract (both potent sources of polyphenols) for the close-up period through 9 weeks in milk. In addition to decreasing liver lipids, supplementation decreased plasma NEFA concentrations after calving and increased milk yield by approximately 10 lb/day in weeks 4 – 8 of lactation. It is not entirely clear that these production responses were due to anti-inflammatory effects of the polyphenols, but they certainly warrant further study.

A different nutritional approach to limiting inflammation is to use omega-3 fatty acids. These polyunsaturated fatty acids have well-described mechanisms underlying their anti-inflammatory effects, although efficiently delivering them to the small intestine is a challenge in ruminants because of ruminal biohydrogenation of dietary unsaturated fatty acids. Nevertheless, feeding whole flaxseed (omega-3 source) compared to sources of omega-6 fatty acids increased plasma glucose and decreased plasma ketones in fresh cows; more surprisingly, the anti-inflammatory omega-3 source resulted in greater phagocytic activity of circulating leukocytes (Gandra et al., 2016). Although this finding of improved metabolic and immune function is exciting, previous studies have reported indications of less responsive immune systems in cows fed omega-3 sources (Lessard et al., 2003; Silvestre et al., 2011), and such findings are more in line with research in rodents. Perhaps the key to beneficial impacts of omega-3 fatty acids on both inflammation and immunity is an improvement in metabolic profile.

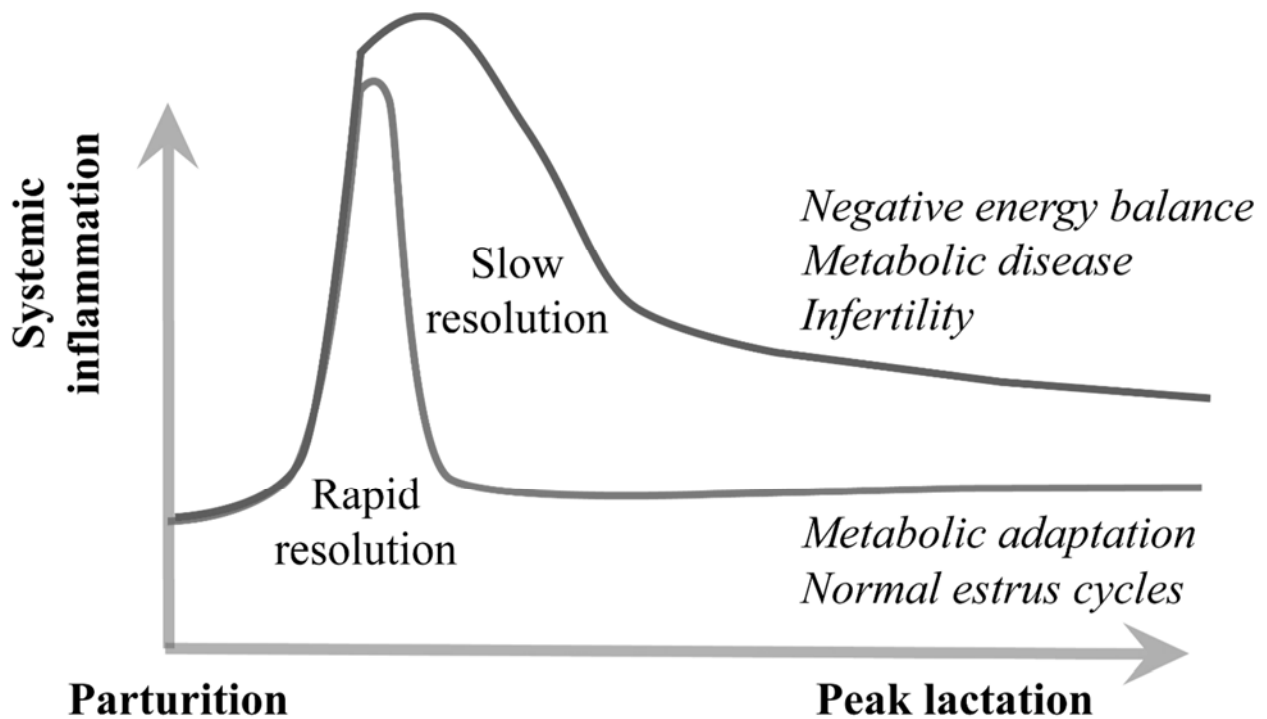
#### IS THERE AN INHERENT CONFLICT BETWEEN PROMOTING IMMUNITY AND PREVENTING EXCESSIVE INFLAMMATION?

Because inflammation is a core component of the immune system's response to an infection, it is logical to ask whether anti-inflammatory strategies may worsen the immunosuppression that is already recognized as a problem in transition cows. In fact, Nightingale et al. (2015) demonstrated that transition cows with the most dramatic inflammatory profiles also had the most potent measures of neutrophil function. One interpretation of these findings is that transition cows are adapted to respond to immunosuppression with a compensatory inflammatory state.

Inherent conflicts between anti-inflammatory strategies and potent immune responses are also suggested by findings of increased infection rates following NSAID treatments in some small studies and greater mortality rates following pathogen challenges in mice genetically engineered to allow for endogenous omega-3 synthesis (Bradford et al., 2015). Likewise, dietary supplementation of an immune modulator resulted in an increased acute phase response to endotoxin (Brandão et al., 2016), suggesting that at least some means of enhancing immunity will likely promote inflammation as well, although conversely, the febrile response was lessened. In support of the concept that a more appropriate balance between immunity and inflammation can be achieved, the immune stimulant described above resulted in increased milk yield (Brandão et al., 2016), and as mentioned before, post-calving meloxicam treatment increased both milk yield and herd retention (Carpenter et al., 2016a).

One question that has not yet been addressed in observational studies is whether the pattern of inflammation impacts long-term outcomes. We hypothesize that brief spikes in inflammatory signals that are resolved in the first 3-4 days of lactation may support immunity and physiological adaptations to lactation. However, failure to rapidly resolve these signals may lead to a variety of adverse impacts that ultimately impair productivity, health, and fertility (Figure 1). We hope that new data will begin to address this question in the coming few years.

Figure 1. Hypothetical impacts of brief, rapidly resolved postpartum inflammation versus sustained inflammation. It is proposed that lack of resolution leads to impaired health and productivity rather than the inflammation *per se*.



### CHOOSING THE RIGHT STRATEGY FOR EACH HERD

In research with anti-inflammatory agents, there have been some marked differences across studies that, while not allowing strong conclusions, hint at predictors for success with these tools. First, treatment with Banamine shortly before and shortly after calving disrupted the normal process of calving and placental expulsion (Newby et al. 2017), resulting in increased incidence of stillbirths (if given before calving) and metritis (if given after calving). This particular approach to combatting calving-associated pain and inflammation is not advised until at least 24 hours after calving. On the other hand, similar strategies with a different NSAID, meloxicam, did not trigger the same negative effects (Newby et al., 2014).

Second, we have seen variable milk production responses to NSAID treatment even when using identical strategies. Treatment with sodium salicylate for 3 days starting 24 hours after calving increased whole-lactation milk yield by more than 2,000 pounds in one study (Carpenter et al., 2016a), whereas in a follow-up study, we observed no milk response at all (Carpenter et al., 2016b). One potentially relevant difference between the cohorts in these two studies is that the responsive group had substantially greater post-calving inflammation, as the mean plasma haptoglobin concentration was more than twice as high in the responsive group compared to the unresponsive group on days 3-4 of lactation. Although we have been unable to demonstrate that individual cows with higher haptoglobin concentrations are more responsive to NSAID treatment, differences between these two studies seem to point in that direction.

Finally, it stands to reason that farms with more infectious disease problems are likely to have more obvious benefits from immune stimulation. As a simple example, on-farm evaluation of responses to a dietary immune modulator showed that decreases in somatic cell count after supplementation began were greatest in herds that started with relatively high somatic cells (Chapman et al., 2016).

Although there is little research basis for this suggestion, mechanisms connecting inflammation and immunity lead to the suggestion that cows in different herds may struggle with different mixtures of transition disorders because of imbalances between pro- and anti-inflammatory signals; excessive inflammation in some herds and inadequate immunity in others. Based on this logic, herds that have relatively high prevalence of infectious diseases in early lactation might be wise to focus on trying immune support tools in an attempt to enhance cows' abilities to combat pathogens. Conversely, herds with more metabolic disorders in early lactation should consider implementing anti-inflammatory management and nutritional strategies. Combinations of both types of supplements may or may not have additive benefits - these interactions simply have not been studied.

## SUMMARY

The growing number of tools available to aid cows successfully transition to lactation is exciting, but, as always, the devil is in the details. Several pharmaceutical and feed additive strategies have strong evidence for specific benefits, but individual farms differ in important ways that can lead to unique questions about secondary effects that are less clear. In particular, unresolved questions about tradeoffs between inflammatory status and immunity make it difficult to give one-size-fits-all recommendations when the transition problems encountered on one farm can differ so dramatically from another. Based on evidence available today, farms with more frequent infectious disease problems are encouraged to explore opportunities to promote immune function, whereas those with prevalent metabolic disorders should perhaps focus more on anti-inflammatory strategies. Research on combinations of such strategies is needed before recommendations can be provided with confidence.



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# IMPORTANCE OF IMMUNE FUNCTION FOR OPTIMAL REPRODUCTION OF DAIRY COWS

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## INTRODUCTION

One of the most widely-discussed issues regarding reproductive management of dairy cows is the historic decline in fertility for lactating cows that began about 1960. It is important to recognize that this situation is being reversed and there has been a steady increase in reproductive function in the last several years (Figure 1). Genetically, the dairy cow is still less fertile than the cow of 50 years ago: the predicted transmitting ability (PTA) of a Holstein bull for daughter pregnancy rate (DPR) in 1965 was +7.1 vs a PTA of +1.4 in 2015. Nonetheless, genetic merit for fertility has been increasing: the nadir for bull PTA for DPR (-1.1) in Holsteins was experienced in 2002. The improvement in genetic merit has involved increased emphasis on health and reproduction traits in leading selection indices like net merit. Fertility has also improved in recent years because of the incorporation of timed artificial insemination (AI) programs in reproductive management systems. Initially, these programs were designed to avoid problems inherent in estrus detection. Now, however, refinements in timed AI programs to optimize the endocrine environment of the cow mean that fertility to timed AI can exceed that following AI at detected estrus (Santos et al., 2017).

While difficult to document, it is likely that increased management emphasis on cow comfort has also contributed to the increase in reproductive function of lactating cows. Indeed, as will be shown in this paper, health is closely connected to resumption of estrous cycles after calving, pregnancy rate per insemination once estrous cyclicity has resumed and maintenance of a pregnancy after a cow is diagnosed pregnant.

The connection between health, immune function and reproductive function is complex and not fully understood. A schematic diagram illustrating some mechanisms by which inflammation and immune activation cause disruption in reproductive function is shown in Figure 2.

Activation of the inflammatory response by local tissue damage (due to injury, ischemia, exposure to noxious molecules, etc.) or by viral or bacterial infection leads to the release of a variety of biologically-active molecules such as cytokines, chemokines, and prostaglandins that assist in tissue repair and promote activation of immune responses that in turn involve synthesis and release of additional cytokines. Biologically-active products of inflammation and the immune system can act at sites distant from the site of tissue injury. For example, specific cytokines affect the hypothalamus to increase body temperature which is damaging to the oocyte and embryo (Hansen, 2014). Additionally, inflammatory signals can reduce appetite and alter metabolism of

carbohydrates, fat and protein (Gifford et al., 2010). Immune responses also increase energy utilization although the magnitude of this increase in cattle is unclear. Resultant changes in energy balance associated with inflammation make it more difficult for cows to resume estrous cycles after calving because the length of the postpartum anestrus is related to energy status (Crowe, 2008). Other actions of cytokines on the hypothalamic-pituitary axis cause inhibition of release of the gonadotropins necessary for establishment and maintenance of estrous cyclicity. Specific cytokines can disrupt the function of the oocyte and developing embryo (see for example, the negative effects of tumor necrosis factor- $\alpha$ ; Soto et al., 2003). Molecules released by microorganisms, particularly those, like bacterial lipopolysaccharides, that activate toll-like receptors on mammalian cells, can act directly on the ovary to affect follicular development and oocyte function (Bromfield and Sheldon, 2011, 2013) and on the endometrium to increase secretion of prostaglandins, cytokines and chemotactic agents that disrupt uterine function and the local environment of the embryo (MacKintosh et al., 2013). Perturbation of the local environment is particularly likely when bacterial infection is ongoing in the uterus itself (Bromfield et al., 2015).

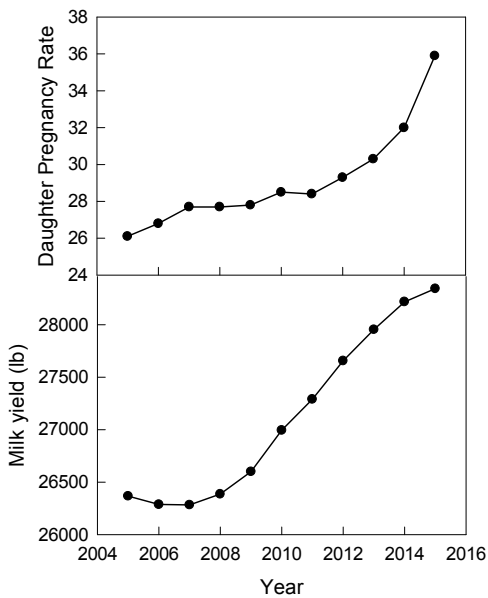


Figure 1. Changes in daughter pregnancy rate and milk yield in US Holsteins from 2005-2015 based on records maintained by the Council on Dairy Cattle Breeding (<https://www.uscdcb.com/eval/summary/trend.cfm>). Note that daughter pregnancy rate is the proportion of a bull's daughters eligible to be pregnant in a 21-day period that are pregnant. It is determined by a combination of the proportion of cows that are bred and the proportion of bred cows that become pregnant.

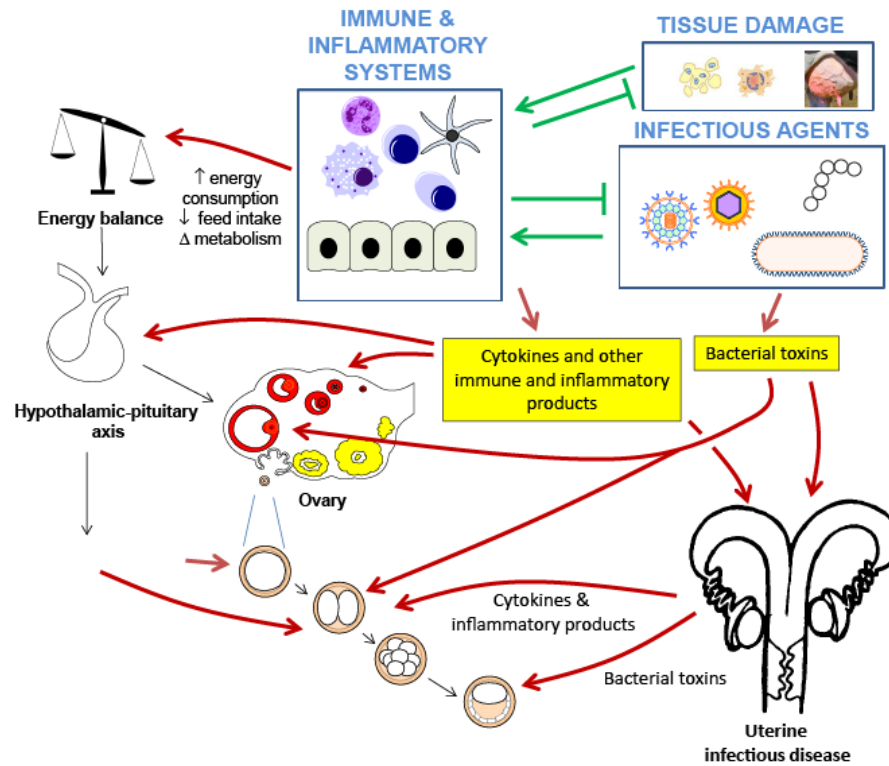


Figure 2. Simplified cartoon to describe some of the mechanisms by which disease and activation of the inflammatory and immune systems can compromise reproductive function in the cow. Red arrows indicate pathways leading to disruption of the reproductive system.

Given the nexus between the immune, inflammatory and reproductive systems, approaches to improve dairy cow health should also result in improved reproductive function. Given this reasoning, there are two goals of the current paper. The first is to show some of the evidence that health and immune function is related to reproductive function. The second goal is to review some recent studies that evaluated prospects for improving cow fertility through regulation of immune function.

### EVIDENCE THAT DISEASE IS ASSOCIATED WITH REDUCED REPRODUCTIVE FUNCTION

Probably the best evidence that a host of disease events compromise reproduction in postpartum cows is derived from studies conducted at the University of Florida by José Santos, Edward Ribeiro, and colleagues. The approach was to classify individual cows as to incidence of specific diseases and then compare reproductive function of cows without disease to those experiencing one or more diseases.

Selected results from two of these studies are summarized in Table 1. In the first experiment (Santos et al., 2010), a total of 5,709 cows were examined. Resumption of estrous cycles, as determined by the percent of cows that were cyclic at day 65 postpartum, was not affected by a single occurrence of disease. However, fewer cows experiencing more than one disease were cyclic at day 65 than cows not experiencing

disease. Pregnancy rate at first AI was reduced if cows experienced one disease and was even lower when cows experienced more than one disease. A wide range of diseases were associated with a significant reduction in reproductive function. For example, pregnancy rate at first AI was reduced from 51.4% in cows without disease to 40.3% for cows with calving problems, 37.8% for cows with metritis, 38.7% for cows with clinical endometritis, 39.8% for cows with fever postpartum, 28.8% for cows with clinical ketosis, and 33.3% for cows with lameness.

Table 1. Association of disease incidents in the postpartum period with resumption of cyclicity and fertility at first insemination.

Study	Number and type of cows	Health status	Endpoint	Value	Adjusted odds ratio	P
Santos et al., 2010	5719 cows on 7 dairies	Healthy	% cyclic at day 65 postpartum	84.1	1.00	--
		1 case of disease		80.0	0.97	0.83
		> 1 case of disease		70.7	0.60	0.001
		Healthy	% pregnant at first AI	51.4	1.00	--
		1 case of disease		43.3	0.79	0.001
		> 1 case of disease		34.7	0.57	<0.001
Ribeiro et al., 2013	957 cows on 2 grazing dairies	No disease	% cyclic at day 49 postpartum	91.1	1.00	--
		1 clinical disease		88.3	0.74	0.22
		> 1 clinical disease		77.8	0.34	<0.01
		No disease	% pregnant at first AI	66.9	1.00	--
		1 clinical disease		56.5	0.64	<0.01
		> 1 clinical disease		40.8	0.34	<0.01

Similar findings were obtained in a second study of 957 cows on two seasonal grazing dairies (Ribeiro et al., 2013). Again, more than 1 clinical disease was associated with reduced cyclicity while even a single clinical disease was associated with reduced pregnancy rate at first AI (Table 1). The diseases associated with reduced pregnancy rate at first AI were calving problems, metritis, clinical endometritis, and digestive problems. There was also a tendency ( $P \leq 0.10$ ) for pregnancy rate to be lower for cows with mastitis or lameness.

In a recent study, Ribeiro et al. (2016) used records from 5,085 cows to evaluate whether the impact of uterine disease (metritis and retained placenta) on postpartum reproductive function was greater than the impact of other diseases (including mastitis, digestive problems, pneumonia, and lameness). The reduction in pregnancy rate at day 45 after breeding (via AI or embryo transfer) and calving rate after the first breeding postpartum was similar for cows with uterine disease vs those with other diseases. Cows that had both types of disease were less fertile than cows that experienced one kind of disease only. Both types of disease also increased pregnancy loss after cows were confirmed pregnant at day 45 after breeding. Santos et al. (2010) and Ribeiro et al. (2013) also noted association of disease with increased pregnancy loss.

Ribeiro et al. (2016) found that disease reduced pregnancy rate for both cows that were bred by AI and those that received an embryo. Thus, at least some of the association of disease with pregnancy failure represents disruption of reproductive function after the early period of pregnancy. In another experiment reported in the same paper, the uterus was flushed at Day 5 or 6 after first AI postpartum to evaluate effect of disease on fertilization of the oocyte and development of the early embryo. Occurrence of uterine disease and other, non-uterine types of diseases were associated with reduced cleavage rate and proportion of embryos classified as live or high quality. Effects of disease on elongation of the embryo at Day 15 and 16 of gestation, and accumulation of the antiluteolytic hormone interferon-tau, in the uterus was also assessed. Previous occurrence of either uterine disease or non-uterine disease were associated with smaller conceptuses and lower accumulation of interferon-tau in the uterine lumen. Taken together, results indicate that disease can affect a wide variety of events in early pregnancy that are required for successful gestation.

#### VARIATION IN IMMUNE FUNCTION IS AN IMPORTANT DETERMINANT OF POSTPARTUM DISEASE

Studies discussed in the previous section provide compelling evidence that a cow that experiences one or more diseases in the postpartum period is at risk for suboptimal reproduction including delayed breeding, reduced pregnancy rate and increased rate of pregnancy loss. Whether or not a cow develops a disease in the postpartum period depends on a variety of factors including the nature of pathogen exposure (number and virulence), function of physiological and nutritional systems important for development of specific diseases like gastrointestinal problems and lameness, and characteristics of the immune system. The importance of the nature of pathogen exposure is evident because while most cows have bacteria present in the uterus after calving, there are differences



in the microbiome of cows whose uterus remains healthy and those that develop metritis or endometritis (Bicalho et al., 2017ab).

Experiments on genetic variation in the function of the immune system have revealed the importance of the immune system for establishment of specific diseases in the postpartum period. In particular, Millard and colleagues at the University of Guelph have used measurements of antibody production and delayed hypersensitivity reactions in the skin to estimate genetic breeding value for antibody-mediated immune function, cell-mediated immune function and overall immune response (based on the first two measurements). For some diseases, including mastitis, displaced abomasum, and retained fetal membranes, cows with low estimated breeding value (EBV) for overall immune response were most likely to experience disease (Table 2).

Table 2. Disease incidence (percent) in cows classified based on estimated breeding value (EBV) for overall immune response (Thompson-Crispi et al., 2012).

Disease	Low EBV for immune response (n=153)	Average EBV for immune response (n=407)	High EBV for immune response (n=139)
Mastitis	25.7 <sup>ab</sup>	29.0 <sup>a</sup>	19.4 <sup>b</sup>
Metritis	7.2	4.7	4.3
Ketosis	5.9	5.7	5.8
Displaced abomasum	5.8 <sup>a</sup>	1.7 <sup>b</sup>	3.6 <sup>ab</sup>
Retained fetal membranes	13.1 <sup>a</sup>	5.9 <sup>b</sup>	5.0 <sup>b</sup>

<sup>ab</sup> Means with different superscripts differ (P<0.05).

There are also indications that numbers or function of polymorphonuclear leukocytes (PMN) in the blood are important for adequate resistance to disease. Cows that subsequently developed metritis or endometritis in the postpartum period had less functional neutrophils than cows that did not develop uterine disease (Hammon et al., 2006). Treatment of periparturient cows with the granulocyte colony stimulating factor (G-CSF) to increase PMN numbers and function reduced the incidence of clinical mastitis (Canning et al., 2017; Ruiz et al., 2017). Similarly, supporting PMN function by feeding OmniGen-AF® reduced incidence of udder edema and tended to reduce incidence of mastitis (Nace et al., 2014).

#### PRELIMINARY STUDIES TO TEST WHETHER ENHANCING IMMUNE FUNCTION CAN IMPROVE REPRODUCTION

Given that postpartum disease is associated with reduced reproductive function and that immune function is one determinant of whether a cow experiences an adverse health effect, it follows that it may be feasible to improve reproductive function in lactating cows by enhancing immune function. There are many potential approaches for doing so including regulating calcium metabolism postpartum (Vieira-Neto et al., 2017), improving energy balance (Lacasse et al., 2017), feeding nutritional supplements that enhance

immune function (Nace et al., 2014) or by provision of biologicals like G-CSF that regulate components of the immune system (Canning et al., 2017; Ruiz et al., 2017).

It is too soon to know whether regulating immune function to improve reproduction will be effective but the idea is worth pursuing because reduction in disease incidence can also have other beneficial effects on the productivity and longevity of the cow. The only large-scale studies on effects of immune stimulants on reproductive function have been performed using G-CSF (Canning et al., 2017, Ruiz et al., 2017). In those studies, treated cows were injected with G-CSF at 7 days before expected calving and again within 24 hours after calving. The effects of this treatment on reproductive function were mixed. In the experiment by Canning et al. (2017), 320 cows per treatment were enrolled (80 cows per treatment at each of 4 dairies). Treatment with G-CSF reduced the incidence of mastitis but not of dystocia, metritis, lameness, pneumonia or a combination of ketosis, left displaced abomasum or peritonitis. A greater percent of cows treated with G-CSF were detected in estrus by day 80 postpartum (95.4% vs 90.6%) but there was no effect of treatment on pregnancy rate at first service (42.6% vs 38.2%). The study by Ruiz et al. (2017) involved 10,238 cows in 17 herds. Incidence of mastitis was reduced by G-CSF but there was no significant effect on incidence of retained placenta. Surprisingly, G-CSF increased incidence of metritis from 8.4 to 9.8%. Cows treated with G-CSF were 5.8% more likely to be inseminated in the first 100 days after calving than control cows.

These studies with G-CSF have not yet made clear whether reproductive function is amenable to change through immune stimulation. It may be that activation of PMN function at other times postpartum or promotion of function of other components of the immune system would be more effective at increasing fertility than that cause by injection of G-CSF or other agents around the time of calving. More studies are warranted.

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# IMMUNITY AND SUB-ACUTE RUMINAL ACIDOSIS

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## INTRODUCTION

Sub-acute ruminal acidosis (SARA) can occur as a consequence of feeding high energy rations to dairy cattle. During SARA, the rate of rumen short-chain fatty acid (SCFA) production exceeds SCFA absorption and results in an unhealthy depression of rumen pH. The severity of SARA is quantified based on the duration and magnitude of depression of rumen pH below a threshold (typically 5.6 or 5.8), and has been most well characterized in ruminally cannulated cows.

Consequences of SARA include depression and fluctuations in intake, reduced diet digestibility, reduced milk yield, reduced milk fat percent, gastrointestinal damage, liver abscesses, and lameness (Krause and Oetzel, 2006; Radostits et al., 2007; Plaizier et al., 2008). Though some of these effects can be remedied with management changes to resolve SARA, localized and systemic inflammation resulting from SARA can cause long term negative impacts on animal health and wellbeing. This review will discuss the impacts of SARA on the digestive tract, inflammation resulting from SARA, and steps that can be taken to reduce SARA.

## IMPACT OF SARA ON THE RUMEN

During SARA, the increase in ruminally fermentable carbohydrates increases SCFA production and leads to shifts in the rumen microbiome. Several studies have used sequencing technologies to evaluate the changes in the rumen microbiome in response to SARA induction or high grain feeding. High grain feeding or SARA has been found to decrease in diversity of both the rumen fluid microbiome and the bacteria adhered to the rumen epithelium (Mao et al., 2013; Petri et al., 2013; Wetzels et al., 2017). When sequencing technologies have been used to characterize the rumen microbiome, at the phylum level, high grain diets tend to increase the relative abundance of *Firmicutes* and decrease the relative abundance of *Bacteroidetes* (Khafipour et al., 2009b; Mao et al., 2013), though effects are not always consistent across studies. The use of PCR to identify changes at the species level has demonstrated that high grain rations or a SARA challenge can result in a decrease in fiber fermenting bacteria including *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* and an increase in *Streptococcus bovis*, *Escherichia coli*, and *Megasphaera elsdenii* (Tajima et al., 2001; Khafipour et al., 2009b; Petri et al., 2013). Khafipour et al. (2009b) found that the increase in *E. coli* was positively correlated with the severity of SARA symptoms, leading them to conclude that increases in *E. coli* may be important to the etiology of SARA. In a follow up study, they found that the concentration of *E. coli* and *E. coli* virulence factors in rumen fluid was approximately 3 logs higher in cows given a grain based SARA challenge known to cause inflammation compared to alfalfa pellet induced SARA that does not trigger an inflammatory response

(Khafipour et al., 2011). Dysbiosis is a term used to describe an unhealthy shift in the bacterial community and has been associated with inflammatory diseases in humans and rodent models (Caesar et al., 2012; Vieira et al., 2013; Kobozev et al., 2014). A similar phenomenon may be occurring in the rumen and intestines as a result of SARA (Khafipour et al., 2016). Collectively, these data suggest that shifts in rumen bacterial communities in response to SARA are a key first step in the negative impacts of SARA on animal performance.

Concurrent with shifts in microbial populations, there is also an increase in rumen concentrations of potentially toxic and inflammatory compounds during SARA. The one that has received the most attention is lipopolysaccharide (**LPS**). Lipopolysaccharide is a component of gram negative bacterial cell walls, and is classified as an endotoxin because the presence of LPS within the body elicits an inflammatory response by mammalian cells. When animals are challenged with a SARA-inducing ration, the availability of fermentable carbohydrates initially results in logarithmic growth of bacteria, which is later followed by massive bacterial lysis in response to reduced availability of substrates, reduced rumen pH, and accumulation of fermentation end products (Zebeli and Metzler-Zebeli, 2012). Free LPS accumulates both during rapid growth and during bacterial lysis, resulting in increased rumen concentrations of LPS during SARA (Li et al., 2012). During an acute acidosis challenge in cows, rumen fluid collected following the challenge had increased endotoxin activity and became increasingly toxic when injected into mice (Nagaraja et al., 1978). These results led the authors to conclude that the effects of acidosis were mediated by systemic effects of rumen endotoxin. In addition, rumen concentrations of LPS were found to be negatively correlated with milk fat percentage and yield when cows were fed increasing levels of barley grain (Zebeli and Ametaj, 2009). Although rumen accumulation of LPS during SARA may be important for subsequent inflammatory responses, the immunoreactive properties of LPS differ among bacterial species, and Khafipour et al. (2009b) propose that the inflammatory response to SARA is due to *E. coli* LPS.

Other potentially harmful compounds produced during SARA include biogenic amines and ethanol (Ametaj et al., 2010). Ethanolamine is a biogenic amine that not only has potentially harmful effects on the host but has also been shown to enhance growth and virulence factor production by pathogenic bacteria (Saleem et al., 2012; Zebeli and Metzler-Zebeli, 2012). Histamine is another biogenic amine produced during SARA and its potential role during the inflammatory response to SARA will be discussed later in this review as it relates to laminitis.

The rumen epithelium serves as a selective barrier, allowing for absorption of SCFA while preventing entry and colonization by bacteria. Systemic effects of SARA are dependent upon a breach in this barrier. Structurally the rumen epithelium consists of four layers, the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale (Figure 1). In the healthy rumen, bacteria are loosely associated only with the stratum corneum. Tight junction proteins that regulate the permeability barrier are expressed most heavily in the stratum granulosum and to some extent in the stratum spinosum (Graham and Simmons, 2005). Connections among the stratum granulosum, stratum spinosum,

and stratum basale allow for the transport of SCFA from the rumen contents to the basal lamina (Graham and Simmons, 2005). The permeability barrier function of the rumen responds to changes in the animal or the rumen. For example, permeability is increased during oxidative stress, heat stress, and feed restriction (Mani et al., 2012; Zhang et al., 2013). Increased permeability may also be an adaptive response to higher grain diets to allow for increased uptake of SCFA (Zebeli and Metzler-Zebeli, 2012). Studies using isolated sections of rumen have also demonstrated increased permeability in response to acidification or hyperosmolality (Schweigel et al., 2005; Emmanuel et al., 2007).

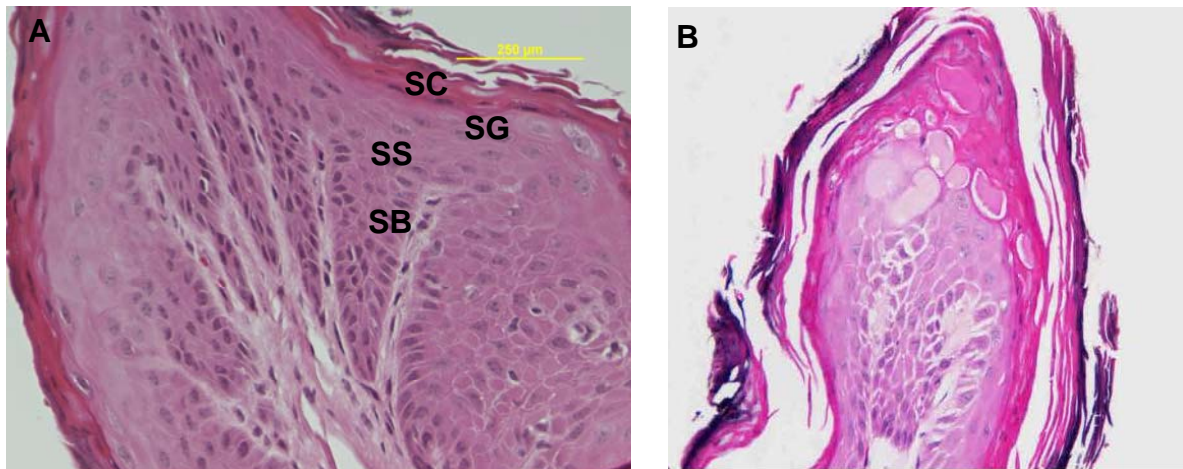


Figure 1. A. Cross-section of a rumen papilla showing the stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). B. Damaged papilla showing separation of stratum corneum.

## CHANGES IN THE DIGESTIVE MUCOSA IN RESPONSE TO SARA

In addition to its role as a selective barrier, the rumen and intestinal epithelia help direct immune function through its interactions with gut-associated lymphoid tissue (**GALT**). These microstructures are found throughout the digestive mucosa and consist of clusters of white blood cells and immune structures in close association with digestive epithelium. They range in size from large Peyer's patches to small isolated lymphoid follicles and consist of clusters of B and T lymphocytes interspersed with dendritic cells and phagocytes (Goto and Kiyono, 2012). Antigen presentation from M cells or dendritic cells causes B cells to be activated to IgA-secreting plasma cells. Secretory IgA then exits the columnar epithelial cells via transcytosis where it accumulates in the mucus to prevent attachment and translocation of target bacteria (Kamada et al., 2013). T cells in germinal centers within GALT are activated by receptor binding of microbial products and locally produced cytokines. In a healthy animal, tolerance of commensal gut microorganisms is facilitated by T cells with a regulatory phenotype that tend to suppress inflammatory responses by surrounding cells (Littman and Pamer, 2011).

Activities of lymphocytes and phagocytes within the gut of a healthy animal respond to the microbiome to elicit appropriate responses: tolerance and local immunosuppression in response to commensal organisms and inflammation and immune activation in response to a pathogenic challenge. Binding of bacterial components to pathogen recognition receptors such as toll like receptors (TLR) and NOD-like receptors is essential for homeostasis. Normal development of GALT structures is dependent upon functional pathogen recognition by these receptors. In addition to promoting proper GALT development, a symbiotic mix of commensal bacteria promotes mucus production and barrier function of the epithelium and inhibits colonization by competitive organisms (Kamada et al., 2013). The effects of the microbiome on GALT and mucosal functions are not only through direct interactions of microbial components with receptors but also through products of symbiotic microbes including short chain fatty acids (Brestoff and Artis, 2013). Dysbiosis can down-regulate these protective functions, stimulate mucosal inflammation, and potentiate colonization by pathogenic organisms.

Downstream inflammatory effects of SARA are dependent on a breach in the permeability barrier of the digestive epithelium. During SARA, some combination of increased osmolality, reduced pH, increased bacterial toxins such as LPS, and increased biogenic amines leads to disruption of the barrier function. A study using isolated rumen and colon tissue from steers demonstrated that LPS and decreased pH acted synergistically to reduce barrier function (Emmanuel et al., 2007). Once the epithelium has been breached, GALT cells may respond by triggering local inflammation and altering cytokine production; this in turn may further increase permeability, potentiate colonization by pathogenic organisms, enhance passage of bacteria and toxins across the epithelium, and increase the inflammatory response (Mani et al., 2012; Kurashima et al., 2013). When cows were switched from a 0% grain ration to a 65% grain ration, the rumen epithelium underwent dramatic changes including visible papillae lesions, decreased tight junctions, sloughing of the stratum corneum, and presence of bacteria in the stratum granulosum and stratum spinosum (Steele et al., 2011). Khafipour et al. (2011) found increased RNA levels of virulence and adhesion factors in *E. coli* isolated from rumen fluid during grain-induced SARA, indicating that SARA may increase the potential for pathogenic organisms to take advantage of a breach in epithelial integrity and colonize papillae.

Concurrent with local inflammation in the papillae are changes in epithelial cell cycle, adhesion protein expression, and SCFA absorption. Compared to cows fed high forage diets, high concentrate diets have resulted in dramatic differences in gene expression, including differences in genes for adhesion proteins and cell cycle regulation (Taniguchi et al., 2010; Steele et al., 2011; Ma et al., 2017). An in vitro study by Meissner et al. (2017) found that exposure of rumen tissue to reduced pH and increased SCFA disrupted epithelial barrier function and reduced expression of the tight junction proteins occludin, claudin-4, and claudin-7. Similarly, a study in goats revealed that a high grain diet decreased the epithelial integrity and the expression of claudin-4 and occludin in the omasum (Liu et al., 2014). Collectively, these studies demonstrate that SARA causes dramatic changes to the rumen epithelium including reduced barrier function. Injury to the rumen epithelium and changes to the cell cycle in response to SARA can result in parakeratosis or hyperkeratosis (Penner et al., 2011). Increased exposure of the more



basal epithelial layers to bacteria and toxins as a result of parakeratosis can further increase rumenitis and lead to the formation of microabscesses (Kleen et al., 2003).

Events that occur in the rumen during SARA are mirrored in the large intestine. An increase in intestinal carbohydrate fermentation typically occurs concurrent with SARA and leads to increased concentrations of SCFA and LPS, a reduction in pH, and damage to the intestinal mucosa (Bissell, 2002; Dijkstra et al., 2012; Li et al., 2012). Fecal indicators of SARA include diarrhea, frothy feces, increased particle size in feces, and presence of mucin casts in feces (Hall, 2002). Because the intestinal epithelium is composed of only a single layer of epithelial cells, it has been proposed that systemic inflammatory effects of SARA might be due to passage of bacteria or toxins through the intestinal mucosa (Oetzel, 2003). In fact, Khafipour et al. (2009a) found that the timing of the presence of LPS in the blood following a SARA challenge suggested LPS entered the circulation via the intestines instead of the rumen. Studies in goats found that compared to feeding high forage diet, a high concentrate ration resulted in evidence of colonic damage including epithelial injury, damaged tight junctions, increased markers of apoptosis, increased inflammatory cell infiltration, and greater gene expression of inflammatory mediators (Tao et al., 2014a; Tao et al., 2014b).

#### SYSTEMIC EFFECTS OF SUB-ACUTE RUMINAL ACIDOSIS

If bacteria or toxins enter the mucosa through a breach in the epithelium, they may trigger localized inflammation, enter the liver through the portal blood supply, or travel systemically through the lymphatics or blood. For example, both SARA and acute acidosis result in increased concentrations of endotoxin in both the hepatic portal vein and hepatic vein (Haubro Andersen et al., 1994; Chang et al., 2015). These results indicate that endotoxin generated in the digestive tract directly enters the liver where it can affect liver function and also exits the liver where it can trigger systemic effects. The presence of endotoxins in the liver, bloodstream, or lymphatic system can then trigger systemic inflammation (Eckel and Ametaj, 2016). For example, Chang et al. (2015) found that expression of inflammatory genes was up-regulated in the livers of cows fed a SARA inducing diet. Increased toxin flow to the liver can result in hepatocyte damage, and Bobe et al. (2004) noted that SARA can increase the likelihood of fatty liver which can further impair liver function. Further, SARA increases oxidative stress in the liver (Abaker et al., 2017), which can damage liver tissue and reduce the ability of the liver to detoxify gut-derived endotoxin. In addition, if live bacteria exit or bypass the liver, they can cause chronic inflammatory diseases in response to SARA such as pneumonia, endocarditis, pyelonephritis, and arthritis (Oetzel, 2007). Bacteria may also colonize the liver and form abscesses. *Fusobacterium necrophorum* is the primary agent isolated from liver abscesses in feedlot cattle, and the liver infection is secondary to infection of the rumen wall (Nagaraja and Chengappa, 1998). This normal inhabitant of the rumen increases in number in response to high grain diets and can opportunistically colonize a rumen wall that has been damaged by parakeratosis or rumenitis in response to SARA (Tadepalli et al., 2009). These are just a few examples of the negative effects of SARA on liver function, and it is likely that other bacterial products and toxins entering the liver as a result of SARA may also impair liver function.

One clear response of the liver to grain-induced SARA is production of acute phase proteins that can modify immune function and generate a systemic inflammatory response. Acute phase proteins include serum amyloid A, haptoglobin, LPS-binding protein, C-reactive protein, and  $\alpha$ -1 acid glycoprotein. Their effects are multifaceted and include stimulating or suppressing an immune response, stimulating tissue repair, removing harmful compounds, isolating infectious agents, and preventing or modifying inflammation (Zebeli and Metzler-Zebeli, 2012; Eckel and Ametaj, 2016). In addition, endotoxins acting directly or indirectly through acute phase proteins can trigger release of inflammatory cytokines by the liver and tissues (Eckel and Ametaj, 2016). Plaizier et al. (2008) summarized results from multiple SARA challenge studies and proposed that LPS, inflammatory amines, or other products of bacteria that reach the liver stimulate release of acute phase proteins from the liver and generate a systemic inflammatory response. Thus, systemic inflammation does not appear to be dependent on bacterial compounds reaching the general circulation. In addition to their release by the liver, mRNA expression of acute phase proteins has also been detected in the gastric mucosa, indicating that the mucosa may contribute directly to this inflammatory response as well (Dilda et al., 2012).

Studies have also been aimed at evaluating why grain-based SARA challenges induce an increase in circulating acute phase proteins while alfalfa-based SARA challenges fail to do so. In a study using cows with ruminal and cecal cannulas, Li et al. (2012) found that although rumen concentrations of LPS increased in response to both types of challenges, cecal concentrations of LPS only increased in response to the grain-based challenge. They propose that translocation of LPS from the large intestine to the liver of grain-challenged animals might account for the increase in acute phase proteins. However, using challenge models that bypassed the rumen, we and others have been unable to generate similar increases in plasma acute phase proteins as found in response to high grain diets, perhaps due to the short-term nature of those challenges (Bissell, 2002; Mainardi et al., 2011). Khafipour et al. (2009b) found that of the microbiome shifts in response to SARA, rumen *E. coli* abundance, which increased only in response to grain-based SARA challenges, was most strongly associated with concentration of acute phase proteins in the blood. These results suggest that differences in bacterial products reaching the liver in response to dietary changes can differentially impact acute phase protein production. Those authors also suggested that increased LPS binding protein concentrations in the blood are a direct indicator of LPS translocation from the rumen to the liver (Khafipour et al., 2009a). As data on acute phase protein response to SARA continues to mount, it is becoming clear that direct passage of LPS or other bacterial products to the general circulation may not be necessary for the systemic inflammatory response to SARA. Instead, immune modulation at the level of the liver or even the gut mucosa seems to be sufficient to drive systemic inflammation.

Laminitis and lameness are consequences of SARA, and it is likely that similar mechanisms to those driving systemic inflammatory responses to SARA also mediate hoof damage. In response to rumen acidosis, vasoactive substances including LPS and biogenic amines can be absorbed across the gut mucosa. Damage to the gut wall and

entry of bacterial products can drive formation of endogenous vasoactive products including cytokines and prostaglandins. The primary effect of these exogenous and endogenous compounds is dilation of arterioles and constriction of venules which at the level of the gut can enhance inflammation and increase entry of toxins (Shearer, 2011; Eckel and Ametaj, 2016). In the corium of the hoof, these vascular changes result in inflammation, hemorrhage, death of cells, activation of matrix metalloproteinases, and disruption of growth factor signaling (Shearer, 2011). Altered cell growth, cell damage, reduced oxygen and nutrient flow, and reduction of intercellular adhesion can cause sinkage of the pedal bone, damage to the corium, pain, and lesions (Nocek, 1997; Goff, 2006). Histamine that is absorbed from the gut or produced endogenously during inflammation has been proposed to play a key role in development of laminitis. In a study using bulls, Takahashi and Young (1981) demonstrated that grain overload and histamine injection to the digital artery acted synergistically to induce laminitis. As reviewed by Katz and Bailey (2012), equine laminitis resulting from starch overload occurs via a similar mechanism to that proposed in ruminants. A loss of barrier function in the gut allows for influx of bacterial products including LPS and amines into the portal circulation. The resulting inflammatory changes in liver and leukocytes, with or without systemic entry of toxins, is proposed to cause laminitis through vascular changes in the hoof, apoptosis, oxidative injury, and enzymatic degradation of the basement membrane (Katz and Bailey, 2012).

## MANAGING COWS TO REDUCE THE IMPACT OF SARA

At the level of the rumen, causes of SARA can broadly be classified as management, environmental, and animal factors which reduce ruminal buffering capacity or increase ruminal SCFA accumulation. As reviewed by Stone (2004), buffering capacity can be increased by increasing dietary forage content and optimizing particle size to increase chewing and saliva flow, by addition of external buffers or alkalinizing agents to the ration, and by increasing the dietary cation anion difference of the ration. Risks of SARA can be reduced by following feeding recommendations including maintaining adequate particle size and physically effective fiber and avoiding excesses of fermentable carbohydrates (Stone, 2004). Buffering capacity can be reduced in response to heat stress or as a result of decreased chewing, for example due to feed sorting. Increasing dietary buffering during heat stress, practicing heat stress abatement, and preventing feed sorting can help to reduce the incidence of SARA. The rate of SCFA production and the risk for SARA can be increased in response to increased dietary proportion of grain, increased fermentability of grains or forages, increased feed intake, and management factors that lead to larger and less frequent meals. Fecal consistency should be regularly monitored, particularly following ration changes, for signs of SARA in the herd. Individual differences in SARA susceptibility may be related to feed intake variation, variation in saliva buffering, variation in SCFA uptake, and differences in endotoxin tolerance (Khafipour et al., 2009b).

Dietary supplements offer the potential to reduce the negative impacts of SARA, either by mitigating events in the digestive tract or by reducing subsequent inflammatory events. Inclusion of live yeast or yeast product can help to stabilize the rumen microbiome

to alleviate the negative effects of SARA (AlZahal et al., 2014). Other direct fed microbials including *Enterococcus faecium* or *Lactococcus lactis* may help to stabilize the rumen environment (Chiquette et al., 2015). Inclusion of feed supplements such as linseed oil or fish oil that contain high levels of omega-3 fatty acids may help to reduce the inflammatory response and tissue damage that can result from feeding high carbohydrate diets (Mani et al., 2012). Other dietary supplements such as biotin and zinc have the potential to strengthen epithelium to prevent tissue injury from SARA (Goff, 2006). A recent study also indicated that thiamine supplementation during a SARA challenge increased rumen pH, decreased rumen LPS, and reduced expression of inflammatory proteins in the rumen epithelium (Pan et al., 2017). The immunomodulatory agent OmniGen-AF (Phibro Animal Health, Teaneck, NJ) has been shown to reduce the systemic inflammatory response to LPS (Brandão et al., 2016), suggesting that it may help to alleviate SARA symptoms by reducing the systemic response to endotoxin derived from the digestive tract. Finally, as we continue to increase our understanding of pathologic bacteria that contribute to SARA-induced tissue damage, there may be potential to develop management strategies to reduce the competitive ability of those organisms. For example, Gill et al. (2000) found that vaccination against *Streptococcus bovis* reduced the severity of response to an acute acidosis challenge in sheep, and future development of vaccines against pathologic bacteria associated with SARA might be beneficial.

## CONCLUSIONS

Sub-acute ruminal acidosis impairs cow performance and health. Rumenitis is the initial insult of SARA and results in inflammatory and immune activation which reduces energy available to support production, allows for transfer of bacterial products across the gut epithelium, and can damage tissues including the liver and hoof. Sub-acute ruminal acidosis will likely continue to be a problem for the dairy industry as high energy diets are required to support high levels of milk production. Careful attention to nutritional management and development of new SARA mitigation strategies may help to reduce its impact in the future.

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## HEAT STRESS EFFECTS ON IMMUNE FUNCTION IN DAIRY CATTLE

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Environmental factors, particularly heat stress, present challenges to dairy cow productivity and health. In the absence of active cooling, exposure to elevated temperatures and humidity significantly reduce dry matter intake during lactation, and subsequent declines in milk yield can approach 25% depending on the severity and duration of the heat stress (reviewed by West, 2003). Lactating cows make metabolic adaptations which repartition additional energy away from productive purposes such that the actual decline in production exceeds what would be expected based on the lower DMI (Rhoads et al., 2009). Similarly, when dry cows are exposed to heat stress they produce less milk than cooled dry cows in the next lactation, even when both groups are cooled after calving (reviewed in Tao and Dahl, 2013). Dry cows under heat stress consume less feed than cooled cows, but the metabolic adaptations observed in lactating cows are not present (Tao et al., 2012). Despite the significant losses associated with productivity under heat stress, additional negative impacts on the overall health of lactating and dry cows and their calves accrue with exposure to high temperatures and humidity, and those outcomes are the focus of this paper.

Udder health is one of the first considerations with heat stress. Heat stress negatively impacts leukocyte migration into the mammary gland after a chemotactic challenge in lactating cows (Elvinger et al., 1992). When lactating cows were subjected to graded increases in temperature and humidity (i.e. Temperature Humidity Index [THI]), leukocyte numbers declined as did circulating cytokines, including TNF-alpha and IL-10, further evidence of a broad effect of heat stress to depress immune status (Zhang et al., 2014). Lactating ewes under heat stress had reduced leukocyte counts versus those provided shade, and had elevated mastitis pathogen loads in their milk relative to shaded flock mates (Sevi et al, 2001). Milk SCC, however, was unaffected by heat stress in ewes, but that may reflect a lack of power to detect differences. In general, heat stress reduces the cow's immune competence.

When ambient temperature and humidity rise, a cow will experience significant shifts in endocrine systems to accommodate to the need to increase heat loss. In particular, cortisol and prolactin, both hormones known to affect the immune system, increase with heat stress (reviewed in Collier et al., 2008). Collier et al. (2008) reviewed evidence that prolactin and cortisol exert influences over genes associated with immune responses, notably the heat shock proteins (HSP's) that act to limit the negative effect of excessive temperatures at the cellular level. Thus, it follows that heat stress would affect immune function at the interface of the endocrine and immune systems.

Heat stress has direct effects on immune function in cows during the dry period, and those impacts linger after calving. For example, adaptive immune function, as measured by the proliferative capacity of lymphocytes, is directly suppressed with exposure to heat stress (do Amaral et al, 2011, 2012). Further negative impacts on adaptive immunity were noted when cows' antibody production was assessed to a foreign antigen in the form of chicken ovalbumin, an innocuous non-self protein that elicits an immune reaction. No difference was observed in antibody production to cows at dry off, but after two weeks exposure to heat stress, antibody production in response to a booster of ovalbumin was reduced relative to that in cooled dry cows (do Amaral et al., 2011). This differential response persisted through the dry period but disappeared after calving when all cows were cooled, further evidence that heat stress directly depressed antibody production. Because many dry cow management protocols include vaccination against mastitis causing pathogens, it is important to consider heat stress abatement as an additional approach to improve health and immune function as the cow transitions into lactation.

One concern regarding dry cow cooling is the observation that the higher milk yield drives greater bodyweight loss in early lactation, and that is associated with greater circulating NEFA and BHBA concentrations as well (do Amaral et al., 2009). Elevated NEFA and BHBA have been associated with reduced immune status, so it is possible that dry cow cooling would lead to reduced immune function after calving. However, we have observed that like milk yield, there is a positive carryover effect of dry cow cooling on innate immune function, as cooled dry cows have more robust neutrophil action relative to those that were heat stressed, despite elevated NEFA and BHBA (do Amaral et al., 2011; Thompson et al, 2014). There is also evidence that pathogen mediated disease incidence is lower in cows that were dry during cool seasons compared with hotter periods (Thompson and Dahl, 2012), further support for the concept of a positive effect of dry cow cooling on subsequent health.

Heat stress can also affect the immune status of the developing fetus, with significant carryover effects after birth. Calves born to heat stressed dams are lighter at birth and typically born 4 to 5 days earlier than those from cooled dams (Tao et al., 2012). In utero heat stress reduces the calves circulating immunoglobulin concentrations during the first month of life, which is negatively associated with health and survival (Tao et al., 2012). A series of studies indicate that the observed reduction in IgG concentrations results from poorer absorption of IgG from colostrum, regardless of the source and quality of that colostrum (Monteiro et al., 2014). Thus, it appears that in utero heat stress alters the calf's ability to absorb IgG. Recent work supports the concept that gut closure is accelerated in heat stressed calves, such that IgG absorption is reduced (Ahmed et al, 2015). As the calf matures, in utero heat stress is associated with higher numbers leaving the herd before completing the first lactation compared with calves from cooled dams, which is consistent with poorer immune status in early life compromising later health (Monteiro et al., 2016).

The most common method of cooling cows during the dry period is consistent with that of lactation cooling, i.e. shade, fans, and soakers to provide active cooling (Collier et al., 2006). In the University of Florida facilities, soakers are positioned over the feedline and fans are over the free stalls. When the ambient temperature exceeds 21.1 °C, fans automatically turn on and the soakers are activated for 1.5 min at 5 min intervals. Using this system, we consistently observe reductions in rectal temperatures of 0.4 to 0.5 °C, and normalization of respiration rate from over 70 breaths/min in heat stressed cows to less than 45 breaths/min in the cooled cows. Most important is the need to cool cows for the entire dry period rather than just the close-up or latter stages. In a recent study we observed a negative impact of heat stress imposed at any time during the dry period on subsequent yield (Fabris et al., 2017a); it is likely that similar negative outcomes occur with regard to immune function.

There may also be nutritional management approaches to ameliorate some of the negative effects of heat stress. Ingredients that improve skin surface exchange of heat through vasodilation, such as niacin, have met with some success in reducing body temperature under heat stress. Supplementation of encapsulated niacin reduced rectal temperatures in lactating cows under heat stress, as a result of greater evaporative heat loss (Zimbelman et al., 2010). However, Lohölter et al. (2013) did not observe an effect of niacin on rectal temperatures, although the form of the niacin or level of heat stress may have affected the response. Indeed, skin temperature increased, but rectal temperature was unaffected in lactating cows supplemented with nicotinic acid (DiCostanzo et al., 1997). Betaine, a natural osmolyte, increased milk yield in lactating cows under thermoneutral conditions but not under heat stress (Hall, et al., 2016). However, betaine did alter heat shock protein (HSP) 27 and HSP 70 expression in leukocytes, with an inverse shift, i.e. decreased HSP 27 and increased HSP 70 following heat stress. Betaine also improved subsequent milk yield when fed to dry cows for the entire dry period (Monteiro et al., 2017). Limited information, however, is available with regard to nutritional modulators of immune status, especially with heat stress.

Feeding one immunomodulatory compound, however, has shown consistent reductions in rectal temperature and respiration rate in heat stressed cows. OmniGen-AF fed at the recommended rate has reduced the rectal temperature of lactating (Lieva et al., 2017), transition (Brandão et al., 2016), and dry cows (Fabris et al., 2017) when animals are housed under heat stress. Whereas the reduction in rectal temperature in dry cows is not as great as that observed with active cooling, there is a substantial lowering of respiration rate as well, and OmniGen-AF feeding before, during and after the dry period to heat stressed cows increased subsequent milk yield (Fabris et al., 2017b).

In addition to the effect on rectal temperatures, OmniGen-AF feeding to lactating cows reduced circulating cortisol during heat stress relative to cows not receiving OmniGen-AF (McBride et al., 2016). As expected, the lower cortisol with OmniGen-AF feeding was associated with improved immune function during heat stress. To further tease out the mechanism of lower cortisol, cows were challenged with corticotropin releasing hormone (CRH) and adrenocorticotropin (ACTH), two of the normal upstream stimulators of cortisol release. During heat stress, responses to CRH and ACTH were

lower in OmniGen-AF fed cows relative to controls. Because cortisol is negatively associated with immune function, the reduction in responsiveness to its physiological stimulators is likely a factor in the immunomodulatory effect of OmniGen-AF.

Consistent with the impact on rectal temperature and respiration rate, OmniGen-AF feeding also positively affected the immune status of heat stressed dry cows. We have hypothesized that immune status early in the dry period is critically important to successful and full involution of the mammary gland and subsequent regeneration of secretory cells for the next lactation (Fabris et al., 2017c). OmniGen-AF feeding in late lactation improved neutrophil function and the expression of L-selectin, which suggest a greater capacity for immune response at dry off. Coupled with the observation that mammary restructuring was altered by OmniGen-AF feeding, and the improved milk yield, this suggests that better immune status early in the dry period may lead to more complete involution and greater regeneration of functional mammary tissue.

In summary, heat stress reduces immune status in lactating and dry cows, and many of these effects are mediated via the immune system that links to altered metabolism and production. Cooling cows throughout the production cycle is critical to optimize health and productivity, and is easily achieved through facilities improvements. Dietary measures may further enhance the positive effects of cooling on lactating and dry cows.

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## THE SCIENCE BEHIND NATURAL ALTERNATIVES IN PRODUCTION AGRICULTURE

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Antibiotic resistance is one of the most challenging public health topics of our time as there is wide-spread concern that antibiotics may no longer cure bacterial infections, and common infections could once again prove fatal to the human population.

The new Veterinary Feed Directive, along with withdrawal of some medications like Neo-Terramycin, has made parts of animal health and production more challenging. Among those challenges, calf rearing is one that stands out as particularly difficult. Calves are threatened by infections over the entire rearing period. Depending on the pathogen, diarrhea occurs at different weeks of life. Often, infections are not treated because antibiotics do not work against viruses and/or bacterial infections, or some medications are no longer permitted. The natural gut defense system is affected for several weeks or months thereafter. The initial step of viral and bacterial protection is a strong innate defense system.

For the last 5 years, Innovad has researched ways of reducing and replacing medication by combining unique natural and non-antibiotic related technologies which can condition the gut, strengthen the barrier junction, minimize the effect of scouring, balance the microflora, reduce inflammatory cytokines, and overall, support faster recovery which enhances the innate animal immune system. Among others, esterified butyrins – molecules composed of a glycerol structure and butyrate – exhibit excellent antibacterial properties along with a high concentration of butyric acid. A very consistent response has been recorded in markets where gut related medication has been banned in calves and other production animals.

On the other hand, **toxins** in their non-polar or conjugated forms are also a challenging topic currently in animal production due to their great impact on cow intestinal health and the immune system. Their presence in feed increases translocation of bacteria, enhances susceptibility to disease, and consequently, impairs **immunity** and overall health and production. Often, due to their (low) concentration in feed, there is not always a clear correlation to clinical signs, making it difficult to identify a toxin challenge. When looking at the bigger picture, we quickly realize that co-contamination, emerging toxins, their modified forms and underestimation of contamination, their effect on bacterial resistance and the difficulty of proper biomonitoring exposure makes possible mitigating strategies complex and interconnected. Knowledge about these modified forms of mycotoxins and the related risk, and most importantly, their true impact on gut health, immunity and overall performance is essential.

Innovad, in collaboration with Ghent University, has embarked on an innovative research program dedicated to **analyzing animal biological fluids** (urine and feces or excreta) that will give explain in which phase I and II metabolites are formed, how long they remain present after exposure, and if they can be linked to the exposure. Combining strategies, with proper technology and understanding opens up new and exciting avenues for mitigating health challenges in a natural, holistic way.



# MANAGEMENT OF FRESH COWS FOR BEST BEHAVIOR

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## INTRODUCTION

Promoting feed intake by lactating dairy cows, particularly those in early lactation, is critical for the improvement and maintenance of milk production and health. Many dairy cows are capable of producing quantities of milk in much greater amounts than which can be maintained by nutrient intake in early lactation. Research in dairy cattle nutritional management has resulted in many discoveries and improvements in dairy cow health and production. Despite many advances this field we are still faced with the challenge of ensuring adequate dry matter intake (DMI) to maximize production and prevent disease, particularly in dairy cows during the early lactation period.

Field observations, in addition to empirical evidence, suggest that housing and management can play as large of a role as nutrition in the performance and health of early lactation dairy cows. Much of that impact is mediated through the effects of those factors on the behavior of dairy cows. This paper will, thus, describe the importance of understanding cow behavior in early lactation and how knowledge in this area of science can be used to evaluate nutritional management and housing strategies. In particular, focus will be on allowing cows the time to perform behaviours they require, dietary transition, feeding management, stocking density, and grouping strategies. It is anticipated that with an improved understanding of the behavioral patterns of these cows, combined with proper nutrition, dairy producers can manage their fresh cows to optimize health and production.

## DO COWS HAVE TIME TO BEHAVE PROPERLY?

A dairy cow has a number of things that she needs to accomplish every day. Dairy cows, fed a TMR and kept in free-stall housing, will spend 3-5 h/d at the feed bunk, 0.5 h/d drinking, 10-13 h/d lying down, 2.5-3.5 h/d outside the pen (milking), and 7-9 h/d ruminating. While every 24-h day should be enough time to allow cows to do these things, we know that any factor which may impinge of the cow's ability to devote her time to those activities may have negative consequences. This is particularly problematic in early lactation, as at calving, feeding, resting, and ruminating activity all decrease, while standing time increases.

Dairy cows are motivated to spend approximately half of their day lying down; Jensen et al. (2005) demonstrated that cows have an inelastic demand for about 12-13 h/d of rest. Other researchers have shown that when opportunities to perform behaviors are restricted, lying behavior takes precedence over eating and social behavior (Munksgaard et al., 2005). Adequate lying time has not only been linked to ensuring

good milk production (Grant, 2004), but prevention of cows spending too much time standing has also been linked to prevention of hoof pathologies (Proudfoot et al., 2010) and resultant lameness. In fact, factors that are linked to encouraging resting time in dairy cows, such as larger, less-restrictive stalls, use of well-maintained, deep-bedding, have all been linked to lower prevalence of lameness (Chapinal et al., 2013). Thus, anything that limits the ability of cows to devote the time she needs to lying down, may have negative consequences.

One of the behavioral challenges that dairy cows face at freshening is the sudden increase in time devoted to milking and being outside of her pen. The more time that cows are required to be outside of their pen and resources (feed, water, rest), they are forced to reduce the amount of time that they devote to things like resting or eating, with consequence. Field studies have shown that cows are often outside of their pens for 4+ h/d (Espejo and Endres, 2007; von Keyserlingk et al., 2012); Espejo and Endres (2007) reported a positive association between the prevalence of lameness in high-producing pens with greater time spent outside the pen. Matzke (2003) demonstrated that mature cows and first-lactation heifers gained + 2 and 4 h/d of rest and 2.3 and 3.6 kg/d of milk when they were outside the pen for only 3 versus 6 h/d.

The feeding behavior of dairy cows is also important factor to consider, as it directly relates to the DMI level of the cow, as well as to her rumen health and digestion. The feed intake of a dairy cow is simply a function of her eating behavior; that is the total DMI (kg/d) of a cow is the result of the number of meals consumed daily (#/d) and the size of those meals (kg/meal). Similarly, the DMI can be expressed as a function of the total time a cow spends feeding per day (min/d) multiplied by the rate (kg DM/min) at which she consumes that feed. Thus, if a cow is to consume more feed, she needs to adjust some aspect of her feeding behavior. In recent analyses, we have demonstrated that gains in DMI may be more consistent by getting cows to spend more time feeding at the bunk, broken up into more frequent meals (Johnston and DeVries, 2015). Thus, maximizing time available to eat, to ensure high levels of DMI, is critical. This is particularly important for fresh cows, who often cannot keep up their nutrient intake in early lactation to match meet production and maintenance demands. An excessive or prolonged drop in DMI after calving may result in non-adaptive negative-energy balance, which may lead to subclinical ketosis (SCK), which is estimated to affect ~40% of dairy cows (McArt et al., 2012).

Maximizing time spent feeding at the bunk, in smaller meals, is also important for keeping the rumen stable, by avoiding large post-prandial drops in rumen pH associated with large meals and resultant risk of sub-acute ruminal acidosis (SARA)(Krause and Oetzel, 2006). Not only how cows eat, but also what they eat is important. Sorting of a TMR by dairy cows can result in the ration actually consumed by cows being quite different from that intended. As result, cows do not consume the predicted levels of effective fiber, thereby increasing the risk of depressed rumen pH (DeVries et al., 2008) and low milk fat (DeVries et al., 2011). Further, imbalanced nutrient intake and altered rumen fermentation, as result of sorting, has the potential to impact the efficiency of digestion and production (Sova et al., 2013).

The importance of devoting sufficient time to rumination should also not be overlooked. Dairy cows rely on the process of rumination to fully digest their food. Rumination serves to assist in the breakdown of particles, which not only also for greater microbial activity, thus increasing the rate of fermentation (Welch, 1982), but also helps assist in passage of material from the rumen. Thus, rumination also contributes to ability of cows to maximize their DMI. Rumination also serves to stimulate saliva production and, therefore, assist in rumen buffering and maintenance of a stable rumen environment (Beauchemin, 1991). While rumination time is largely dictated by the diet consumed (and its amount), factors which influence the daily activity patterns of cows have the potential to influence rumination. Dairy cows typically ruminate in a diurnal pattern during the time periods when the animal is not active (feeding, milking), but when at rest (lying down). As such, most rumination activity occurs at night, with other major bouts of rumination occurring during the middle of the day in-between other periods of activity (DeVries et al., 2009). As result, a disruption to a cow's normal rest time, due to other factors (for example: poor stall comfort or availability, increased need to walk, activity related to social agitation) may result in a decrease in rumination time.

#### WHAT ARE THE BENEFITS OF MONITORING BEHAVIOR?

Given the link between feeding behavior and DMI, there is evidence that monitoring feeding behaviors may be important for the detection of health problems in dairy cows. In work by Goldhawk et al. (2009) cows diagnosed with SCK during the week after calving showed differences in feeding behaviour and DMI at the time of diagnosis. Interestingly, those differences were apparent as early as 1 wk before calving. Those researchers estimated that for every 1 kg decrease in DMI and 10 min decrease in feeding time during the week prior to calving, the odds of developing SCK increased by 2.2 and 1.9 times, respectively (Goldhawk et al., 2009).

Another behaviour which may be important to monitor during the transition period is rumination behavior. Shorter rumination times may be indicative of low DMI (Clement et al., 2014), and risk of negative energy balance, during the post-fresh period. For example, Calamari et al. (2014), studying a small group of cows (n=23), reported that cows that were diagnosed with at least one clinical disease postpartum had a lower rumination time in the first week after calving and their increase in rumination time after calving was slower compared with healthy cows. In a larger study by Liboreiro et al. (2015), cows diagnosed with SCK had reduced rumination time from calving to 8 d postpartum, as compared with healthy cows. In a recent study by our group, we demonstrated that multiparous cows who developed SCK, not only had reduced rumination time during the first weeks after calving, but also during the week prior to calving, compared to those cows that remained healthy (Figure 1; Kaufman et al., 2016). These differences were accentuated in those cows that not only were diagnosed with subclinical ketosis, but also with one or more other health problems post-partum.

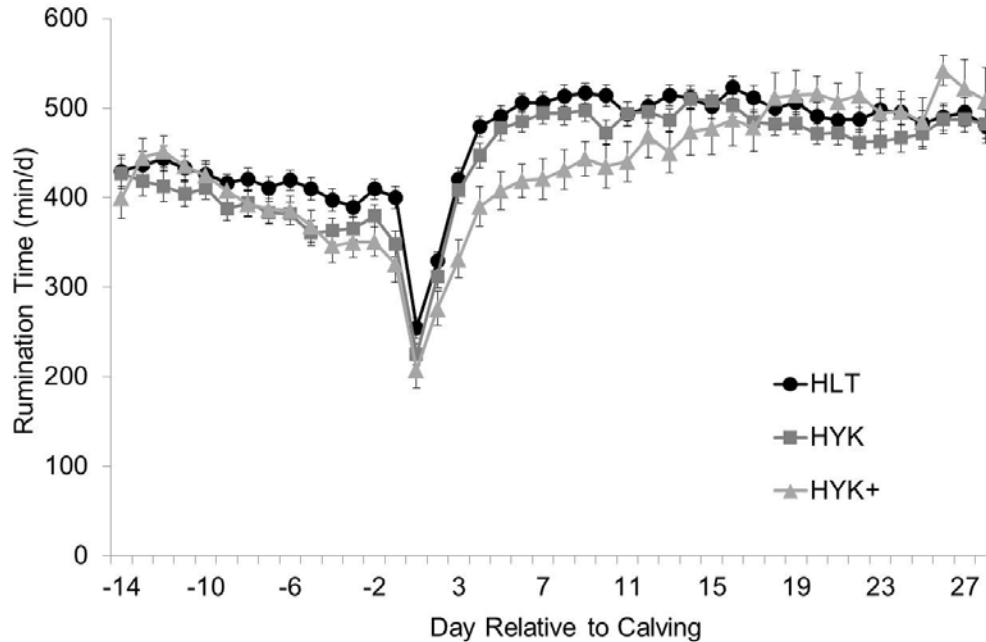


Figure 1. Daily rumination time over the transition period for multiparous cows that were: healthy with no other recorded illnesses (HLT; n = 87), subclinically ketotic with no other health problems (HYK; n = 76) and subclinically ketotic with other health problems (HYK+; n = 39) (adapted from Kaufman et al., 2016).

The results of these studies suggest that careful monitoring of cow behavior in the post-fresh period, as well as before calving, may be useful for identifying cows experiencing illness, or even at risk for illness. This is becoming a reality on many dairy farms with the development, validation, and commercialization of various technologies to automatically capture such behavioral changes (Schirmann et al., 2009; Bikker et al., 2014). Our ability to identify cows at risk for illness maybe even greater on farms with automated (robotic) milking systems, where data from those systems can be combined with that generated from other behavioral monitoring technologies (King et al., 2017).

#### HOW DOES DIET AFFECT BEHAVIOR IN EARLY LACTATION?

One of the most notable changes for the dairy cow at calving is the transition from the dry to lactating diet. It is well established that cows take anywhere from 7 to 14 days to adjust their DMI in response to a dietary change (Grant et al., 2015). Given the difference in composition of dry cow and fresh cow diets, an associated lag in DMI is not always surprising. The susceptibility of dairy cows to SARA is also highest in early lactation (Penner et al., 2007), but also highly variable between cows, despite similar feeding management and transitioning strategies (Penner et al., 2007). Moving from a high-forage dry cow diet to a lower forage, higher NFC fresh-cow diet will not only directly impact the rumen environment, but have impacts on the eating behavior of cows. It is plausible that some of variability may be due to the eating behavior of said diets in early lactation. As compared to eating a dry cow diet, a fresh cow diet will be consuming much faster, in larger meals (DeVries et al., 2007). Such diets are also

sorted to a greater degree (DeVries et al., 2007; 2008) and, as result of lower fibre content and particle size, ruminated for shorter periods of time per unit of feed consumed. Therefore, formulations for fresh cow diets should be aimed at minimizing these impacts on the eating behavior of the cows, by providing adequate physically-effective fiber, while limiting the use of highly fermentable starch sources.

Given that fresh cow diets still require a significant amount of highly-fermentable feed sources to ensure sufficient DMI and to meet nutrient requirements, other opportunities to modify the feeding patterns and rumination of cows on such rations need to be explored. Feed additives that have a positive impact on the rumen environment can also have concurrent benefits for feeding and rumination behavior. We demonstrated that supplementing peak-production lactating cows with a live strain of *Saccharomyces cerevisiae* yeast had beneficial impacts on meal patterning (DeVries and Chevaux, 2014); cows had more frequent meals that were smaller and occurred closer in time together. This research supported previous work by Bach et al. (2007) whereby similar effects on feeding behavior were seen as well a positive impact on raising and stabilizing rumen pH. In DeVries and Chevaux (2014), cows supplemented with live yeast tended to ruminate longer and have less periods of elevated rumen temperature, which could be associated with less long bouts of depressed rumen pH. Likely, as result of these improvements in nutrient flow, rumination, and stabilized rumen, the live yeast-supplemented cows tended to have higher milk fat content and yield. Yuan et al. (2015) demonstrated that feeding a yeast culture-enzymatically hydrolyzed yeast product to cows during the dry period and early post-partum period has similar impacts on feeding behavior, with dry cows having more frequent, smaller meals.

Similar results have been demonstrated with other feed additives – including monensin. Lunn et al. (2005) demonstrated that providing monensin increased meal frequency in lactating cows experiencing sub-acute ruminal acidosis. Similarly, Mullins et al. (2012) found that feeding monensin in the first few days after dairy cows were transitioned to a lactation ration resulted in increased meal frequency and decreased the time between meals.

The common thread in all of these studies is an association between favorable meal patterns and a reduction in ruminal pH variation. Whereas meal patterning may, in itself, affect ruminal pH, it is likely that feed additives, such as live yeast or monensin, that have the potential to stabilize ruminal pH and fermentation, affect meal patterning as a secondary effect. Specifically, a more consistent fermentation pattern should result in less variation in volatile fatty acid production, improved fiber digestibility, and quicker return to eating. Feed additives that promote healthy eating patterns and have a positive impact on the rumen environment and rumination are then particularly useful for early lactation cows, which are at greater risk of experiencing SARA. For these cows, the use of such additives, in addition to proper feed bunk management (as described below), will allow cows to optimize the potential of the feed provided to them and remain healthy and productive during this critical period of time.

## HOW FEED AVAILABILITY AFFECTS BEHAVIOR?

Beyond the diet provided, management of fresh cows must be focused on stimulating eating activity to help cows meet their lactational demands. In a series of studies we have shown that for TMR-fed dairy cattle, feed delivery acts as the primary stimulant on their daily feeding activity patterns (DeVries et al., 2003; DeVries and von Keyserlingk, 2005; King et al., 2016). Therefore, the frequency and timing of delivery of fresh feed are an important factor for stimulating intake in fresh cows.

More frequent feed delivery (than 1x/d) results in cow more evenly distributing their intake across the day (DeVries et al., 2005; Mantysaari et al., 2006) as well as improve access to fresh feed by subordinate cows (DeVries et al. (2005). Further, providing more than 1x/d has been demonstrated to reduce the amount of feed sorting (DeVries et al., 2005; Sova et al., 2013), which would further contribute to more consistent nutrient intakes over the course of the day. Such desirable feeding patterns are conducive to more consistent rumen pH, which likely contributes to improved milk fat (Rottman et al., 2014); fiber digestibility (Dhiman et al., 2002); and production efficiency (Mantysaari et al., 2006) observed when cows are fed more frequently than 1x/d. Improvement in DMI (Hart et al, 2014) and milk production (Sova et al., 2013) are also possible with more frequent feed delivery, however, less expected.

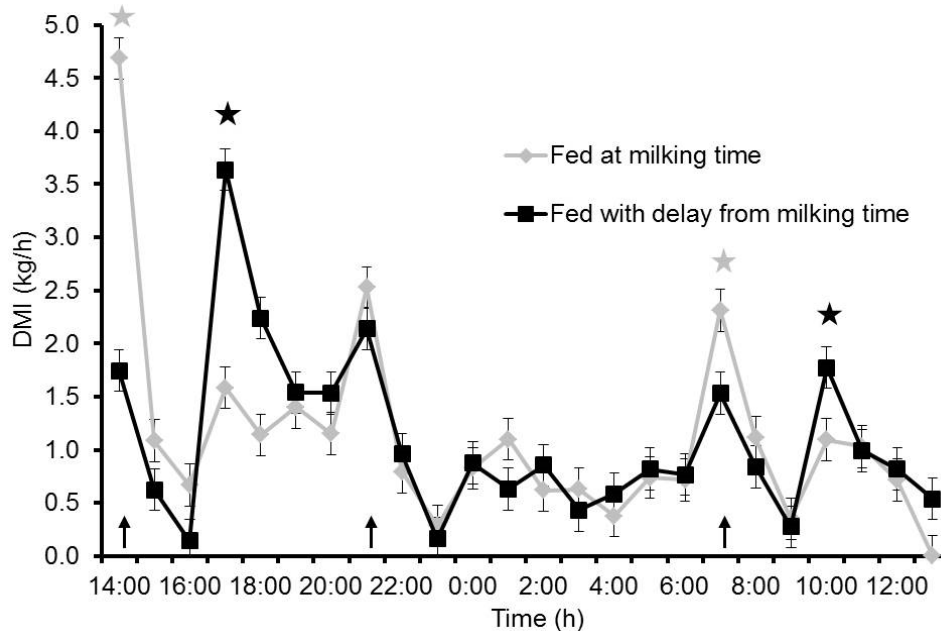


Figure 2. Hourly average DMI (kg) of lactating dairy cows fed 2x/d: 1) at milking time (at 1400 and 0700 h, denoted with ★) or 2) fed with delay from milking time (at 1730 and 1030 h, denoted with ★). Cows were milked 3x/d at 1400, 2100, and 0700 h (denoted with ↑) (adapted from King et al., 2016).

While moving to more frequent feed delivery may be difficult to operationalize on some farms, there is potential to alter the timing of feed delivery to increase the distribution of feed intake across the day. While the delivery of fresh TMR has the greatest impact of stimulating feeding activity, cows are also prone to eat around the time of milking, as well as around other management events during the day. It is possible then to stimulate more meals across the day by staggering these management events, for example, by moving the time of feed delivery away from milking. King et al. (2016) recently shifted feed delivery (2x/d) ahead of milking (3x/d) by 3.5 h and found that this resulted in cows consuming their feed more slowly in smaller, more frequent meals across the day (Figure 2), improving the efficiency of milk production.

Feed push up is another important factor in ensuring feed availability throughout the day. It must be noted, however, that we have no research evidence to say that feed push-up has the same stimulatory impact on feeding activity as does the delivery of fresh feed (DeVries et al., 2003). There is also no scientific evidence to suggest that pushing up feed more frequently will stimulate more DMI. That being said, feed push up needs to occur frequently enough such that any time a cow decides to go to the feed bunk, there is feed available to her at that time. This ensures that DMI is not limited. By mixing up the feed that is no longer in reach, pushing it up will also help minimize the variation in feed consumed. Thus, pushing up feed frequently is necessary, particularly in the first few hours after feed delivery, when the bulk of the feeding activity at the bunk occurs.

## DO COWS HAVE SPACE TO BEHAVE PROPERLY?

One of the key components to ensuring that cows devote the proper amount of time to the behaviors they need to perform each day is to provide them adequate access to the resources they desire (i.e. feed, water, and lying areas). This is particularly true given that dairy cattle are allelomimetic, that is, they like to perform similar behaviors at the same time (i.e. synchronized). When dairy cattle are overcrowded (i.e. situations where there are more cows than available feeding and/or lying spaces), they do not simply shift their eating and lying patterns to accommodate, but rather reduce the time they devote to those activities.

There are several studies where a reduction in lying time associated with lower stall availability has been described. For example, Fregonesi et al. (2007) demonstrated that increasing stocking density from 100 to 150% (1.5 cows per stall) reduced lying time by ~2 h per day. Similarly, Krawczel et al. (2012) demonstrated that for cows averaging 13 h/d of lying at a stocking density of 100%, increasing free-stall and feed bunk stocking density simultaneously from 100 to 142% resulted in a decrease of lying time of 42 to 48 min per day (Krawczel et al., 2012). Reduced lying time associated with overcrowding forces cows to spend more time standing on potentially hard, wet floors, which is tough on hoof health and may increase risk of lameness (Westin et al., 2016). Further, overcrowding may lead to reductions in rumination behavior. Krawczel et al. (2012) demonstrated that increasing free stall and headlock stocking density from 100 to 142% resulted in a drop of rumination time by 0.4 h/d; this change in rumination was

associated with more time spent ruminating while standing and less time spent ruminating while lying down. These may all cumulate, then, in reduced milk production; Bach et al. (2008) demonstrated in a cross-sectional study of 47 herds, all with similar genetics and feeding the exact same TMR, a positive association ( $r = 0.57$ ) between the stalls/cow and milk yield.

Similarly, overcrowding at the feed bunk results in increased aggressive behavior, and may limit the ability of some cows to access feed at times when feeding motivation is high, particularly after the delivery of fresh feed (DeVries et al., 2004; Huzzey et al., 2006). As a result, increased feed bunk competition will increase feeding rate at which cows feed throughout the day, resulting in cows having fewer meals per day, which tend to be larger and longer (Hosseinkhani et al., 2008). Feed bunk competition may also force some cows to shift their intake patterns by consuming more feed later in the day after much of the feed sorting has already occurred. Alternatively, reducing feed bunk competition, by providing adequate feed bunk space, particularly when combined with a physical partition (e.g. headlocks or feed stalls), will improve access to feed, particularly for subordinate dairy cattle (DeVries and von Keyserlingk, 2006; Huzzey et al., 2006). This, in turn, will contribute to more consistent DMI patterns, both within and between animals, as well as promote healthy feeding behavior patterns. It is, thus, not surprising that Sova et al. (2013), found in a cross-sectional study of parlor-milked, free-stall herds in Canada that every 4 inch [10 cm]/cow increase in bunk space (mean = 21 inch/cow; range = 14 to 39 inches/cow) was associated with 0.06 percentage point increase in group average milk fat and a 13% decrease in group-average somatic cell count. With greater bunk space available, cows are able to consume their feed in a manner more conducive to maintaining stable rumen fermentation, and thus have greater milk fat production. This may be particularly important for early lactation cows, which as described above, are at greatest risk of experiencing SARA during this time period. Also, with more bunk space (and lying space) cows are not forced to choose to lie down too quickly after milking rather than compete for a feeding or lying spot (Fregonesi et al, 2007), and thus reduce their risk of intramammary infection from environmental pathogens (DeVries et al., 2010). Finally reduced feed bunk space has also been linked to compromised reproductive performance (Caraviello et al. 2006; Schefers et al., 2010). To date, much of work on the research on transition cows has been focused on available feed bunk space during the close-up pre-partum period, where it has been shown that limiting bunk space can limit DMI (Proudfoot et al., 2009) and increase risk of post-partum disease (Kaufman et al., 2016). There is little research on this factor for the fresh-cow pen, however, given the vulnerability of cows at this time period, it is expected that these effects may be magnified at this time period. Thus, every effort should be made to manage fresh cow pens to provide sufficient space for all cows to each simultaneously (i.e. 30 inches [0.75m] of bunk space per cow).

In addition to access to feed and lying spots, some consideration must also be given to another, typically forgotten, nutrient: water. Water is perhaps the most important nutrient, however its quality and availability is often overlooked. Interestingly, in a recent field study of free-stall herds Sova et al. (2013) found that milk yield tended



to increase by 0.77 kg/d (1.7 lb/d) for every 2 cm/cow increase in water trough space available on the study herds (mean: 7.2 cm/cow; range: 3.8 to 11.7 cm/cow). While cause and effect were not established in that study, this result highlights the importance of water availability for group housed cows and provides further evidence that resource availability has the potential to greatly impact productivity.

## HOW DO GROUPING AND PEN MOVEMENT AFFECT BEHAVIOR?

The optimal grouping of cows, particularly in the post-fresh period, remains a question. Over the years there have been a number of studies highlighting the differences in behavior of first-calf heifers as compared to mature cows. Krohn and Konggaard (1979) found first-calf heifers housed in a free stall separately from mature cows had increased eating time and higher DMI. Phillips and Rind (2001) reported that a mixed group of first-calf heifers and mature cows on pasture grazed for less time than either parity group kept alone. Most recently, Neave et al. (2017) found that, as compared to mature cows, first-calf heifers in mixed-parity groups spent more time feeding, ate more slowly, visited the feed bunk more frequently, explored their feeding environment more, lay down more frequently in shorter bouts, and were replaced at the feeder more often. Given these differences, there appears to be benefits in keeping first-calf heifers and mature cows in separate groups. Phelps (1992) reported that first-calf heifers kept in groups produced 729 kg more milk per lactation than those kept in groups mixed with mature cows. Bach et al. (2006) observed first-calf heifers housed alone, as compared to those mixed with mature cows, to experience lesser loss of bodyweight and greater efficiency of milk production during the first part of lactation, as well as to milk more frequently in a robotic milking system. In a study done on commercial herds, Østergaard et al. (2010) found that keeping separate first-lactation heifers groups after calving (for one month) positively affected production and health (with reduced treatments of ketosis) in those animals. Based on these data, it is recommended that first-calf heifers and mature cows are housed separately in early lactation to ensure optimal health and production of those first-lactation animals. However, due to herd size and facility design, this is not always possible. This was recently highlighted in a study by Espadamala et al. (2016) of 45 large herds in California, where ~50% of the herds did not keep first-calf heifers in separate groups. For those herds that do not, or are not able to, keep separate groups, it is important for those co-mingled groups for there to be sufficient lying, feeding, and water space, and the lying stalls are designed to fit the largest animals in the pen.

Another important factor to consider in relation to grouping of fresh cows, in the frequency and timing of moving animals into new groups (relocation). It is well established that every time a cow is moved into a new pen it can disrupt the social complex of the group and have specific negative impacts on the moved individual. The negative effects of relocation can be seen for up to 3 d following placement in a new pen, and include increased competition for feed access, greater feeding rate, and reduced production, DMI, and rumination time (von Keyserlingk et al., 2008; Schirmann et al., 2011). Torres-Cardona et al. (2014) also demonstrated that relocation can reduce milk production on the day of relocation, with a greater impact on first-lactation heifers

compared to mature cows. Interestingly, Talebi et al. (2014) demonstrated that the negative effects of relocation can be reduced by decreasing the stocking density of the pen being introduced into. Further, Tesfa (2013) demonstrated that lactating cows, introduced into new groups of cows as pairs, showed no drop in milk production as seen in previous studies. Therefore, for fresh cows, which inevitably will be moved into a new pen at calving, and potentially again later into another lactating cow pen, steps should be taken to minimize the impacts of such relocation. Examples of this include not overcrowding pens, potentially moving cows with familiar companions, or moving cows into new pens during quieter times of the day (away from time of management events, such as feeding or milking).

## SUMMARY

Housing and management play a significant role in the performance and health of fresh cows. Much of the impact is mediated through the effects of those factors on the behavior of dairy cows. Dairy cows need the time and availability of resources to perform those behaviors which are not only important for them, but also for maintaining good production and health. Fresh cow diets should be formulated to maximize eating time and DMI, while minimizing sorting. Management of that feed should be focused on maximizing opportunities for cows to go the bunk across the day, either by increasing the frequency of feed delivery or by altering the timing of feed delivery, while pushing up feed continually between feedings to ensure constant access. Overcrowding during be avoided for fresh cow pens, so that cows can maximize their eating and lying opportunities. Further, keeping first-lactation heifers in separate groups, as well as minimizing group changes, helps decrease social stress. Finally, behavioral monitoring during the post-partum period may also be important for identification of health issues in early lactation, and also for the evaluation of herd-level management strategies and events.

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# **CAN GENOMICS OF DRY MATTER INTAKE IN TRANSITION COWS IMPROVE HEALTH AND FERTILITY?**

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## **INTRODUCTION**

The periparturient transition period in dairy cows encompasses the 6-8 weeks of late pregnancy and early lactation. Parturition results in an abrupt metabolic shift from nutrient accrual (fetus and body reserves) to rapid mobilization of lipid and protein stores for energy metabolism in support of milk production. The cascade of metabolic and physiological changes in the periparturient cow affect productivity, health and future reproductive performance.

In high genetic merit cows, feed intake and energy balance may begin decreasing prepartum. In turn, lower pre-calving dry matter intake (DMI) has been implicated as a risk factor for ketosis and metritis after calving. The onset of lactation after calving is associated with a prolonged period of negative energy balance (NEB) during which energy intake lags behind the energy requirements of rapidly increasing milk production. Lower DMI also affects subsequent reproductive function in that cows with lower voluntary intakes during the final weeks of pregnancy and greater NEB post-calving express delays in their resumption of estrous cycles in early lactation. Thus, levels of energy intake, re-initiation of ovarian activity in advance of the breeding period, and health status are intertwined in transition cows.

During the past decade, adoption of genomic selection in breeding programs by the major dairy-producing countries has led to significant changes in the worldwide dairy industry. When added to the routine evaluation for milk production and other economic traits, genomic selection has markedly accelerated genetic gains. Since sufficient DMI is important for high-producing cows to maintain health and to cover the demands of milk production, interest has increased for genomic prediction of DMI in dairy cattle within overall breeding goals. The purpose of this paper is to explore current information and future potential on the genomics of DMI in relation to successful transition from pregnancy to early lactation.

### **Prepartum Differences in DMI Associated with Cow Health**

Metabolic adaptations in the periparturient cow are both dynamic and complex with the conditions changing daily. An emerging issue is that the pattern and timing of decreased DMI before calving is predictive of upcoming health disorders and perturbations in liver function. Cows that developed metritis (Huzzey et al., 2007) or ketosis (Goldhawk et al., 2009) after calving had lower daily DMI during the two weeks

prior to calving and spent less time feeding. Thus, changes in animal behavior and DMI occurred prior to calving and before any evidence of pathology post-calving.

In cows diagnosed with uterine infection (metritis), associated increases after calving in plasma haptoglobin, an acute phase protein, have been reported (Huzzey et al., 2009; Schneider et al., 2013). Haptoglobin is produced by the liver and is a marker of hepatic metabolic disturbance in response, for example, to inflammation of the uterus and the resultant secretion of cytokines. Cytokines are proteins secreted by white blood cells and tissues during inflammation as a response to irritation or injury caused by infection or other damage (Lor et al., 2013). The various cytokines can be classified as being pro-inflammatory cytokines (e.g. IL-6 and TNF $\alpha$ ) or anti-inflammatory cytokines (e.g. IL-10). In the liver, pro-inflammatory cytokines rapidly promote the secretion into blood of positive acute phase proteins e.g. haptoglobin, whereas, the production and release of other blood proteins such as albumin decreases *i.e.* albumin is a negative acute phase protein (Schneider et al., 2013 and Figure 1).

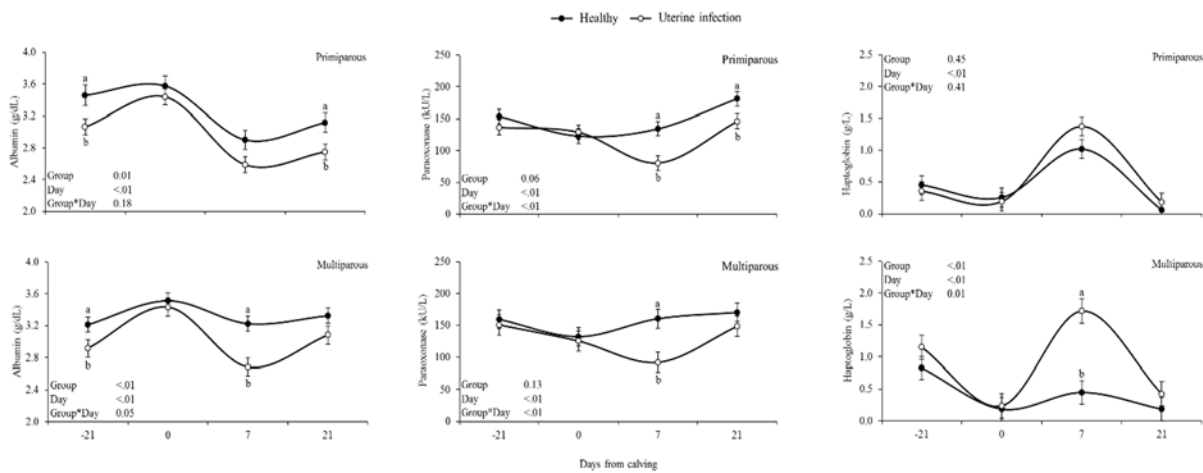


Figure 1. Serum concentrations of albumin (g/dL), paraoxonase activity (kU/L), and haptoglobin (g/L) in healthy cows or cows diagnosed with metritis during the transition period. Different letters indicate differences between groups.

A response to inflammation in postpartum cows can be monitored in terms of both positive and negative acute phase proteins and associated changes in other blood metabolites that allows for calculation of a liver functionality index (LFI; (Trevisi et al., 2012). Low LFI values are indicative of a high inflammatory response and increased pro-inflammatory cytokine release. Low LFI cows were much more likely to encounter disease or health problems after calving as well as having lower DMI and milk production. In cows that develop metritis after calving there is evidence of disturbance in liver function (e.g. acute phase responses -  $\downarrow$  albumin and  $\uparrow$  ceruloplasmin) already occurring during the last 3 weeks prepartum (Schneider et al., 2013; Trevisi et al., 2012).

The periparturient period is characterized by a sudden increase in energy requirements for lactation superimposed on declining voluntary DMI and this results in NEB. An important adaptation to NEB during the transition to early lactation is the



mobilization/lipolysis of fat from body adipose stores with release of non-esterified fatty acids (NEFA) into the circulation. NEFA provides an important source of energy during early lactation when the majority of available glucose is being spared for lactose synthesis in the mammary glands. However, continuous or excessive lipolysis promotes conversion of NEFA into liver triacylglycerol's that can negatively impact hepatic glucose production. The consistent responses in liver to lipid infiltration are reduction in the expression of genes and proteins associated with ATP production and up-regulation of markers of inflammation (e.g. IL-6; (Loor et al., 2007; Loor, 2010).

As described above, inflammation during the periparturient period has emerged as an important aspect of transition cow biology and an excellent review has recently appeared (Bradford et al., 2015). The presence of an inflammatory state in the postpartum period has been documented and the acute phase response, a key secondary response to inflammation, has been well established. The acute phase protein haptoglobin was shown to be elevated in plasma around calving, even in cows that were apparently healthy, but cows with health problems or calving difficulties had higher concentrations (Qu et al., 2014; Schneider et al., 2013 -- see figure1). Elevated haptoglobin levels have been associated with enhanced innate immune responses in white blood cells *i.e.* a systemic inflammatory state (Nightingale et al., 2015), however, the postpartum inflammatory state in many cows is low-grade without the classical signs of inflammation. Bradford et al. (2015) refer to this latter situation as subacute inflammation or metabolic inflammation that is associated with tissue malfunction *e.g.* liver function. In the postpartum period, exposure to high concentrations of fatty acids can disrupt intra-cellular endoplasmic reticulum (ER) membranes in the liver and cause a stress response (Ringseis et al., 2015). Lipid peroxides are also potential mediators that link elevated NEFA to hepatic inflammation. Enhanced peroxisomal oxidation of fatty acids contributes reactive oxygen species (ROS) that increase lipid peroxide formation with the potential for local cell and tissue injury.

#### Peripartum Changes in DMI Associated with Reproductive Performance

By way of various metabolic factors and hormonal interactions, NEB shifts the course of ovarian activity early postpartum and strongly influences the resumption of ovulatory ovarian cycles *i.e.* postpartum interval in days to first ovulation. With regard to fertility to AI during the breeding period, there is a strong positive association between early commencement of ovulatory cycles and pregnancy during lactation (Butler, 2000; Galvao et al., 2010). Following calving, ovarian follicle development and function were monitored for comparison, retrospectively with differences in prepartum DMI, NEB, and metabolic conditions. Cows that achieved full follicular function and successfully ovulated by 3 weeks of lactation had consistently higher prepartum DMI in the last 3-4 weeks before calving, their energy balance was higher (albeit still negative postpartum), and blood NEFA concentrations were lower as compared with cows that developed large nonovulatory follicles and had delayed first ovulation (Butler et al., 2006; Cheong et al., 2016) **See figure 2**. Overall, NEB is minimized in cows that maintain higher DMI until the day of calving and then rapidly increase their intakes over the first several weeks of lactation.

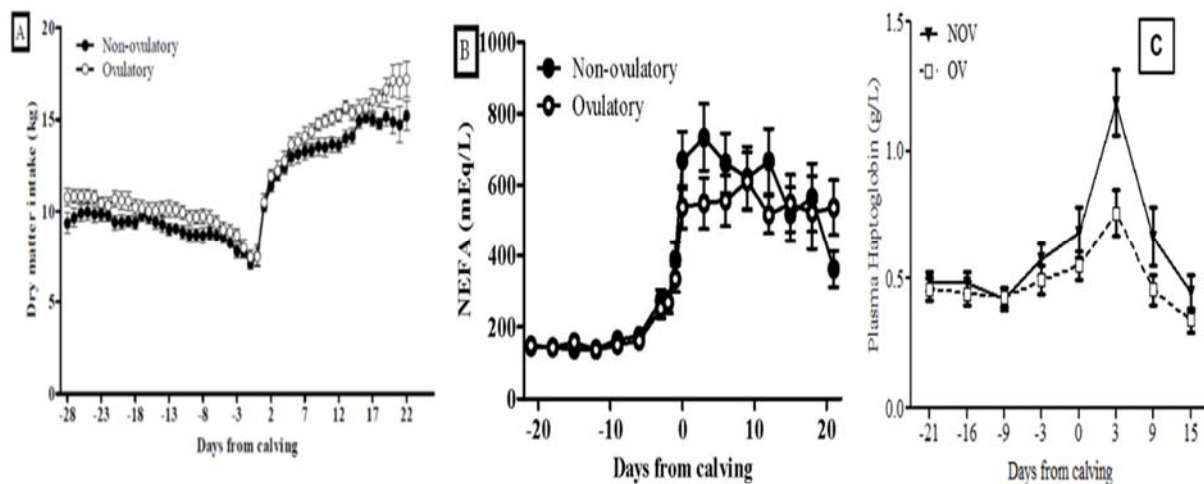


Figure 2. Comparison of the patterns of DMI (kg/d) and plasma concentrations of NEFA ( $\mu\text{Eq/L}$ ) and haptoglobin (g/L) during the transition period in dairy cows with postpartum ovulatory vs. nonovulatory dominant follicles. DMI was consistently higher and plasma levels of NEFA and haptoglobin were lower in cows having large follicles that successfully ovulated within 3 weeks after calving.

Increases in blood NEFA concentrations prior to parturition provide a monitoring tool to assess future impacts on reproduction. In a large field study involving 100 dairy herds, cows with NEFA  $\geq 0.27$  mEq/L in plasma during the week before calving subsequently had 19% lower probability for pregnancy after the start of breeding (Ospina et al., 2010). Cows with greater loss of body condition score loss associated with NEB in early lactation have lower fertility later during the breeding period (Santos et al., 2009).

As presented earlier, the periparturient period in cows is associated with reduced DMI, increased mobilization of NEFA from adipose tissue and a variable degree of liver acute phase response. A strong acute phase response to systemic inflammation characterized by an activated immune system and cytokine response e.g. high plasma concentrations of haptoglobin and  $\text{TNF}\alpha$ , respectively, resulted in impaired reproductive efficiency and longer delays for cows to conceive during lactation (Nightingale et al., 2015). Higher plasma haptoglobin concentrations were also present in cows that developed nonovulatory large dominant follicles after calving (**see figure 2C**).

How might postpartum inflammatory conditions in conjunction with NEB exert carryover effects on fertility in dairy cows? A very interesting and comprehensive report involving large numbers of cows appeared recently (Ribeiro et al., 2016). Cows that were diagnosed with any inflammatory disease before AI had reduced pregnancy rate/AI; **either uterine disease or nonuterine disease** reduced pregnancy rate/AI and their effects were additive. Most interestingly, the occurrence of disease at preantral or antral stages of follicle/oocyte development had similar detrimental effects on pregnancy rate. This indicates that the carryover effects of inflammation can last longer than 12 weeks *i.e.* the estimated time period required for ovulatory follicle development in cows. Thus,

the authors concluded that reduced oocyte competence is the likely reason for the long term detrimental carryover effects of postpartum inflammation.

### Genomic Analyses for Associations with DMI in Lactating Dairy Cows

DMI in dairy cows has moderate heritability and, thus, provides a potentially useful target for genetic selection (Liinamo et al., 2012; Spurlock et al., 2012). Identifying genetically superior animals for DMI is difficult and requires having many animals with phenotypic and genotypic data. As an example of one research approach, the initial goal is to identify genomic regions (areas of chromosomes) significantly associated with DMI. DNA samples from individual cows can be genotyped using beadchips with 50K or 770K single nucleotide polymorphism (SNP) markers for comparison with phenotype information collected on DMI during specific stages of lactation. Genome-wide association studies (GWAS) are then conducted to statistically link markers or groups of markers on specific chromosomes with changes in DMI. The basic approach in GWAS is to evaluate the association between each genotyped marker and a phenotype of interest evaluated in a group of individuals. The results can be depicted in Manhattan plots showing markers or peaks of markers that meet or exceed the statistical criteria for significance (threshold line). An example of a Manhattan plot for DMI at 30 days of lactation is shown in **figure 3A** (Tetens et al., 2014).

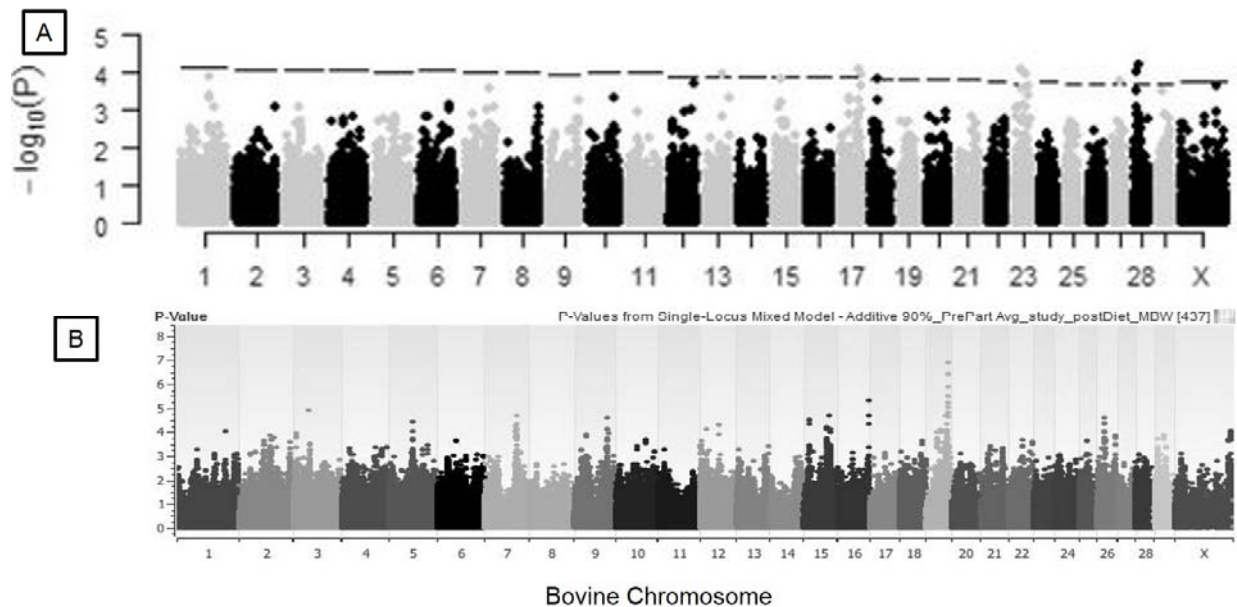


Figure 3. Results of genome-wide association studies (GWAS) depicted in Manhattan plots with chromosomal location of markers associated with DMI. **A)** Data for 30 days of lactation (Tetens et al., 2014). The horizontal line indicates the threshold for chromosome-wise significance. **B)** Data for the first 2 weeks of lactation using 90% as the cut-off threshold for daily energy intake as % of required (Butler et al., unpublished).

In the GWAS study shown in figure 3A, DMI was monitored in primiparous cows during intervals from 11-80 DIM. Significant markers of DMI were found on 8 of the 30 bovine chromosomes (#13, 15, 17, 23, 25, 27, 28, and 29). Using a similar approach in research trials at Cornell University, a GWAS analysis was conducted for a phenotypic variable associated with DMI: *daily dietary energy intake as a % of total energy required* during the first 2 weeks of lactation. This variable was chosen rather than NEB (Liinamo et al., 2012) in order to identify individual cows that best met their energy requirements *via* higher voluntary feed intake and requiring less adipose lipid mobilization. Using a cut-off level of 90% for the high *versus* lower intake cows, the Manhattan plot for genomic areas associated with the phenotype is shown in **figure 3B** (Butler, unpublished). All cows were multiparous and remained healthy postpartum. The 8 chromosomes having the highest concentrations of markers associated with *energy intake as a % of requirement* were: #3, 5, 7, 12, 15, 16, 19, and 26. Obviously, there appears to be little concordance in the genomic regions identified for association with DMI in figure 3. A previous study suggested that lactating cows express their genetic potential for feed intake and energy utilization most clearly between weeks 2 to 10 of lactation (Liinamo et al., 2012) and this has focused our recent research attention.

A second approach for genomic analysis related to DMI in lactating cows is to identify specific candidate genes in physiological pathways relevant to DMI. Researchers may study genes that have shown effects on DMI in previous studies. The objective is to compare the phenotype data among animals with their genotypic polymorphisms *i.e.* do cows having the different alleles (*e.g.* AA, AB, or BB) in the gene show differences in their DMI performance? There are ample examples of differences in the DNA code resulting in differences in the amino acid sequence of the protein product from the gene that may alter phenotype. One example of the candidate gene approach was an earlier study reporting associations between a SNP in the growth hormone receptor gene (GHR) and differences in DMI in dairy cattle (Banos et al., 2008).

GWAS for DMI can also identify potential candidate genes for future research. Once the SNP markers associated with DMI are mapped to specific genomic regions on the various chromosomes, public databases containing information on the bovine genome can be interrogated to find genes in close proximity to the markers. Biological information on any effects of these genes that may be associated with regulation of the phenotype is then assembled for further studies and confirmation. Veerkamp et al. (2012) identified candidate genes for feed intake linked to insulin, cellular growth factors and tryptophan. In another example, adrenergic receptor and leptin genes were identified as candidates to affect DMI from among a list of many other genes in the same chromosome region (Hardie et al., 2017). In the recent Cornell studies, the SLC37A1 gene was highly associated with DMI in early lactation. The protein product of this gene translocates glucose-6-phosphate from the cytoplasm into the lumen of the ER for hydrolysis into glucose by another ER membrane protein. This gene is a member of the solute carrier 37 gene family. Although difficult and expensive, further research will continue to investigate the manner by which multiple genes and their genotype combinations can better predict effects on DMI.

In addition to many research studies investigating the genomic associations directly with DMI in lactating cows, there has also been interest regarding dietary energy utilization and feed efficiency. In a recent study, gross feed efficiency (GFE), defined as the ratio of total energy corrected milk to total DMI, was measured during the first 150 DIM along with energy balance (Spurlock et al., 2012). The genetic correlation between GFE and energy balance ranged from -0.73 to -0.99, indicating that selection for more dietary energy efficient cows would favor a lower energy status. This negative relationship between GFE and energy balance means that higher efficiency cows are deriving more of their energy requirement for milk production from body tissues. Indeed, another study showed that cows who had higher feed efficiency during lactation had greater days open (lower fertility) -- the genetic correlation between GFE and days open was 0.53 (Vallimont et al., 2013). Thus, results from both studies indicate that selection for improved feed efficiency must be carefully considered in order to avoid potential negative consequences on health and fertility associated with greater NEB in early lactation.

## CONCLUSIONS

1. During the periparturient period in cows, changes in DMI, health status, and re-initiation of ovarian activity are intertwined.
2. The periparturient period in cows is characterized by reduced DMI, increased mobilization of NEFA due to NEB, and a variable degree of liver metabolic disturbance/acute phase response.
3. Differences in peripartum DMI among cows are associated with differing effects on ovarian function and subsequent reproductive performance.
4. Genomic analysis has demonstrated that DMI is a complex trait associated with many genomic regions and individual genes.
5. Genomic tools hold promise for better genetic selection for DMI in dairy cows to improve health and reproduction by minimizing NEB.

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# FATTY ACID NUTRITION OF THE FRESH COW

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## INTRODUCTION

Recently, the effects of individual fatty acids (FA) on digestibility, metabolism, and production responses of dairy cows has received renewed attention. The addition of supplemental FA sources to diets is a common practice in dairy nutrition to increase dietary energy density and to support milk production. The ability to understand and model FA, the effects of individual FA, and different FA supplements on production parameters has direct impact on dairy industry recommendations and the usefulness of FA supplementation strategies. In fresh cows, the high metabolic demand of lactation and reduced DMI during the immediate postpartum period result in a state of negative energy balance. Approaches to increasing energy intake of postpartum cows include increasing starch content of the diet and supplementing FA to increase the energy density of the diet. However, feeding high starch diets that promote greater ruminal propionate production during early lactation could be hypophagic and therefore further reduce DMI and increase the risk of ruminal acidosis and displaced abomasum (Allen and Piantoni, 2013). Regarding supplemental FA, some authors suggest that caution should be exercised when using dietary FA to increase the caloric density of diets in early lactation dairy cows, since a high lipid load may affect the endocrine system, feed intake, and increases the risk for metabolic disorders (Kuhla et al., 2016). However, just as we recognize that not all protein sources are the same it is important to remember that not all FA or FA supplements are the same. We will briefly review the biological processes and quantitative changes during the metabolism of FA, the digestibility of these FA, and their overall impact on performance. Our emphasis in the current paper is on recent research supplementing palmitic (C16:0), stearic (C18:0), oleic (*cis*-9 C18:1), omega-3, and omega-6 acids on feed intake, nutrient digestibility, milk production and milk composition, health, and reproduction.

## EFFECTS OF C16:0, C18:0, AND *CIS*-9 C18:1 ON FATTY ACID DIGESTIBILITY

Our recent FA digestibility research has utilized and focused on C16:0, C18:0, *cis*-9 C18:1. Of particular importance, Boerman et al. (2017) fed increasing levels of a C18:0-enriched supplement (93% C18:0) to mid-lactation dairy cows and observed no positive effect on production responses, which was likely associated with the pronounced decrease in total FA digestibility as FA intake increased (Figure 1A). Similarly, Rico et al. (2017) fed increasing levels of a C16:0-enriched supplement (87% C16:0) to mid-lactation dairy cows and even though a positive effect was observed on production response up to 1.5% diet DM, a decrease in total FA digestibility with increasing FA intake was observed (Figure 1B). However, considering that the range in FA intake was similar across both studies, the decrease in total FA digestibility was



more pronounced when there was increased intake/rumen outflow of C18:0 rather than C16:0. This is supported by our meta-analysis, in which a negative relationship between the total flow and digestibility of FA was observed, with the decrease in total FA digestibility driven by the digestibility of C18:0 because of the negative relationship between duodenal flow and digestibility of C18:0 (Boerman et al., 2015). The exact mechanisms for these differences in digestibility are not understood; however, potential causes include the lower solubility of C18:0 compared to C16:0, which would be more dependent of emulsification for absorption (Drackey, 2000). Additionally, results have shown that *cis*-9 C18:1 has greater digestibility than C16:0 and C18:0 (Boerman et al., 2015). Freeman (1969) examined the amphiphilic properties of polar lipid solutes and found that *cis*-9 C18:1 had a positive effect on the micellar solubility of C18:0. To further understand what factors influence FA digestibility, we utilized a random regression model to analyze available individual cow data from 5 studies that fed a C16:0-enriched supplement to dairy cows. We observed that total FA digestibility was negatively impacted by total FA intake, but positively influenced by the intake of *cis*-9 C18:1 (unpublished results). Finally, we recently evaluated the effects of varying the ratio of dietary C16:0, C18:0, and *cis*-9 C18:1 in basal diets containing soyhulls or whole cottonseed on FA digestibility. We observed that feeding a supplement containing C16:0 and *cis*-9 C18:1 increased FA digestibility compared with a supplement containing C16:0, a mixture C16:0 and C18:0, and a non-fat control diet. The supplement containing a mixture C16:0 and C18:0 reduced 16-, 18-carbon, and total FA digestibility compared with the other treatments (de Souza et al., 2016a). This is displayed in Figure 2 by using a Lucas test to estimate the apparent digestibility of the supplemental FA blends. The slopes (i.e., digestibility of the supplemental FA blends) in soyhulls based diets were 0.64, 0.55 and 0.75 and in cottonseed diets were 0.70, 0.56 and 0.81 for supplements containing C16:0, a mixture C16:0 and C18:0, and a mixture of C16:0 and *cis*-9 C18:1, respectively. This supports the concept that a combination of 16-carbon and unsaturated 18-carbon FA may improve FA digestibility, but reasons for this need to be determined.

In fresh cows, there is scarce information about the effects of supplemental FA on FA digestibility. We recently conducted a study to evaluate the effects of timing of C16:0 supplementation on performance of early lactation dairy cows (de Souza and Lock, 2017b). We observed a treatment by time interaction for C16:0 supplementation during the fresh period (1 – 24 DIM); although C16:0 reduced total FA digestibility compared with control, the magnitude of difference reduced over time (Figure 3). Interestingly, we also observed an interaction between time of supplementation and C16:0 supplementation during the peak period (25 – 67 DIM), due to C16:0 only reducing FA digestibility in cows that received the control diet in the fresh period. This may suggest an adaptive mechanism in the intestine when C16:0 is fed long-term. Understanding the mechanisms responsible for this effect deserves future attention, as does the impact of other supplemental FA during early post-partum on FA digestibility and nutrient digestibility.

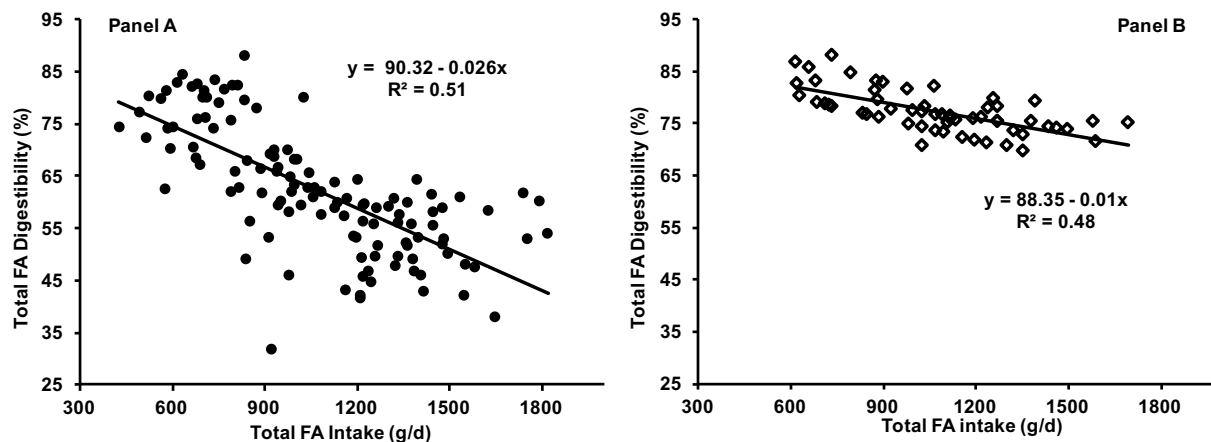


Figure 1. Relationship between total FA intake and apparent total-tract FA digestibility of dairy cows supplemented with either a C18:0-enriched supplement (Panel A) or a C16:0-enriched supplement (Panel B). Results in Panel A utilized 32 mid-lactation cows receiving diets with increasing levels (0 to 2.3% dry matter) of a C18:0-enriched supplement (93% C18:0) in a 4 X 4 Latin square design with 21-d periods (Boerman et al., 2017). Results in Panel B utilized 16 mid-lactation cows receiving diets with increasing levels (0 to 2.25% dry matter) of a C16:0-enriched supplement (87% C16:0) in a 4 X 4 Latin square design with 14-d periods (Rico et al., 2017).

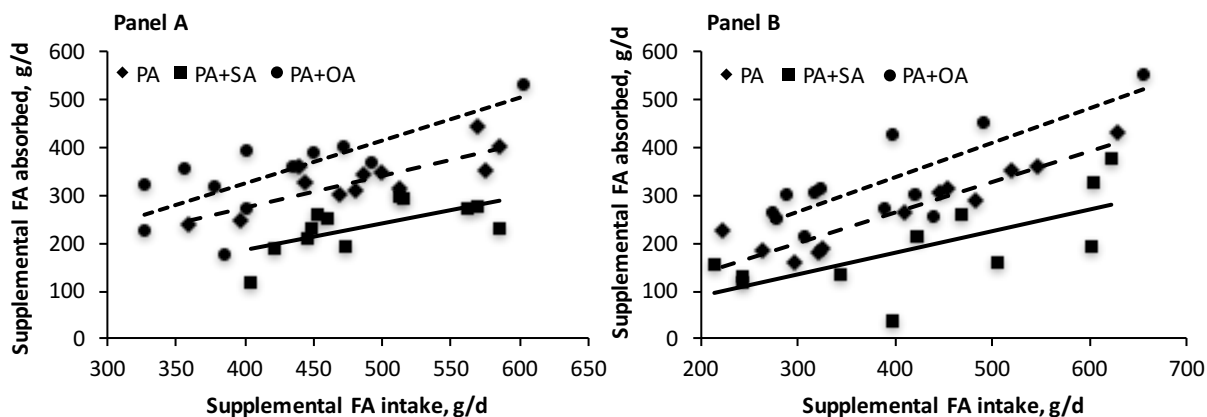


Figure 2. Lucas test to estimate total FA digestibility of supplemental FA treatments when cows received either a soyhulls basal diet (Panel A) or a cottonseed basal diet (Panel B). PA long-dashed line (1.5% of FA supplement blend to provide ~ 80% of C16:0); PA+SA solid line (1.5% of FA supplement blend to provide ~ 40% of C16:0 + 40% of C18:0); and PA+OA short-dashed line (1.5% of FA supplement blend to provide ~ 45% of C16:0 + 35% of C18:1 cis-9). Digestibility of supplemental FA was estimated by regressing intake of supplemental FA on intake of digestible supplemental FA. The mean intakes of FA and digestible FA when cows were fed the control diet were subtracted from the actual intakes of total FA and digestible FA for each observation.

## EFFECT OF FATTY ACIDS ON NDF DIGESTIBILITY

Changes in intake and digestibility of other nutrients, such as NDF, due to FA supplementation may affect positively or negatively the digestible energy value of any FA supplement. Weld and Armentano (2017) performed a meta-analysis to evaluate the effects of FA supplementation on DMI and NDF digestibility of dairy cows.

Supplementation of supplements high in medium chain FA (12 and 14-carbons) decreased both DMI and NDF digestibility. Addition of vegetable oil decreased NDF digestibility by 2.1 percentage units, but did not affect DMI. Also, feeding saturated prilled supplements (combinations of C16:0 and C18:0) did not affect DMI, but increased NDF digestibility by 0.22 percentage units. Overall, the authors concluded that the addition of a fat supplement, in which the FA are 16-carbon or greater in length, has minimal effects on NDF digestibility, but the effect of C16:0-enriched supplements were not evaluated.

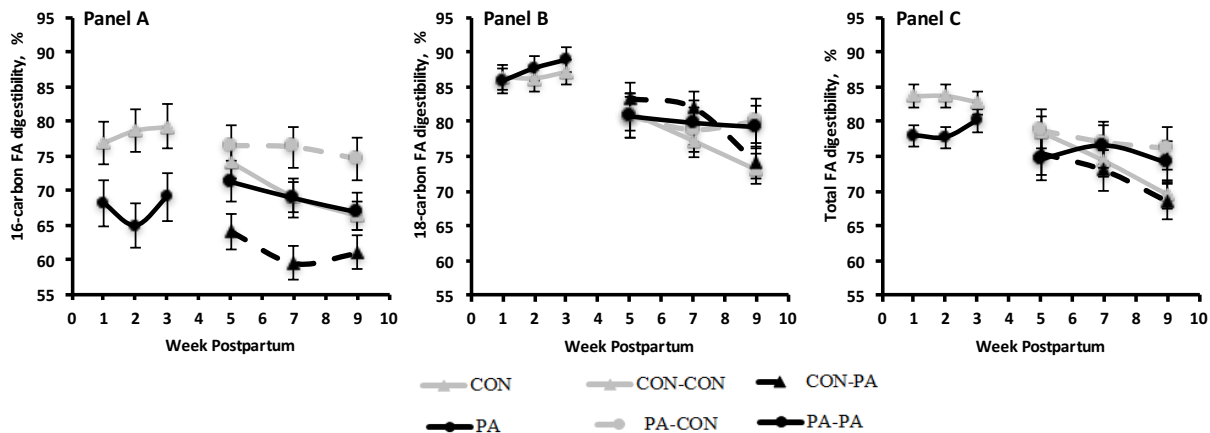


Figure 3. The effects of C16:0-enriched supplementation for early lactation cows on digestibility of 16-carbon (Panel A), 18-carbon (Panel B), and total FA (Panel C). Results utilized 52 early-lactation cows receiving the following diets: no supplemental fat (CON) or a C16:0 supplemented diet (PA) that was fed either from calving (1 to 24 DIM; fresh period FR) or from 25 to 67 DIM (peak period). From de Souza and Lock (2017b).

We recently utilized a random regression model to analyze available individual cow data from 6 studies that fed C16:0-enriched supplements to dairy cows (de Souza et al., 2016b). We observed that NDF digestibility was positively impacted by total C16:0 intake (Figure 4A) and DMI was not affected. This suggests that the increase in NDF digestibility when C16:0-enriched supplements are fed to dairy cows is not explained through a decrease in DMI. Additionally, when comparing combinations of C16:0, C18:0, and *cis*-9 C18:1 in supplemental fat, we observed that feeding supplements containing C16:0 or C16:0 and *cis*-9 C18:1 increased NDF digestibility compared with a supplement containing C16:0 and C18:0 (de Souza et al., 2016a).

With early lactation cows, Piantoni et al. (2015b) fed a saturated fat supplement (~ 40% C16:0 and 40% C18:0) and observed that fat supplementation increased NDF digestibility by 3.9% units in the low forage diet (20% fNDF), but had no effect in the high forage diet (26% fNDF). In our recent study that evaluated the effects of timing of C16:0 supplementation (PA) on performance of early lactation dairy cows (de Souza and Lock, 2017b), we observed that C16:0 supplementation consistently increased NDF digestibility ~ 5% units over the 10 weeks of treatment compared with control (Figure 4B).

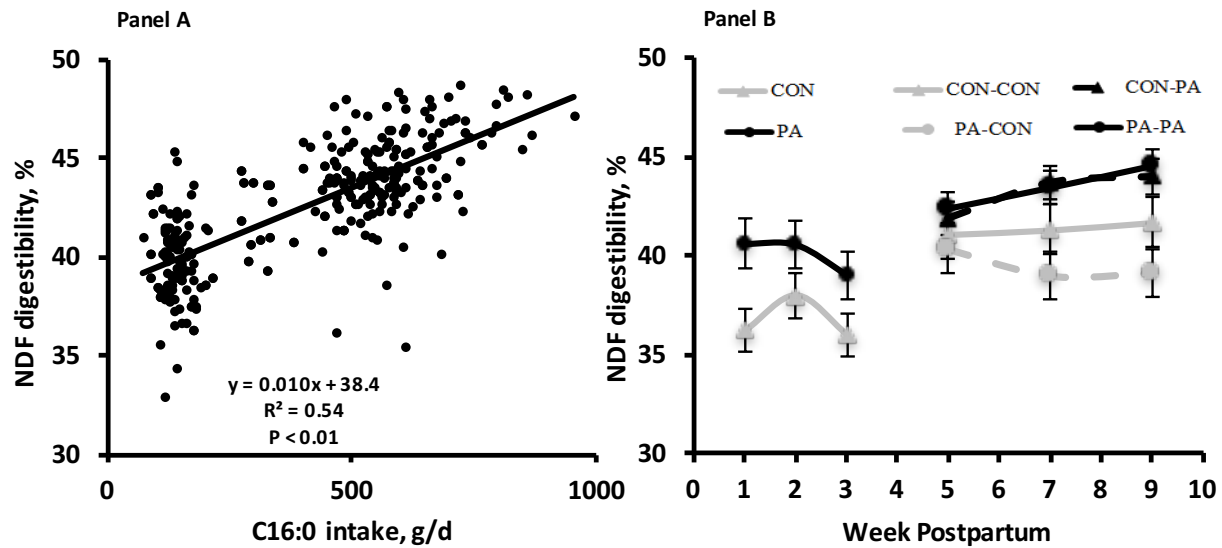


Figure 4. Panel A: Relationship between C16:0 intake and NDF digestibility of dairy cows fed C16:0-enriched FA supplements. Panel B: The effects of C16:0-enriched supplementation in early lactation cows on NDF digestibility. Results in Panel A represent a combined data set evaluated using a random regression model from 6 studies feeding C16:0-enriched supplements on NDF digestibility of post-peak cows (de Souza et al., 2016b). Results in Panel B utilized 52 early-lactation cows receiving the following diets: no supplemental fat (CON) or a C16:0 supplemented diet (PA) that was fed either from calving (1 to 24 DIM; fresh period) or from 25 to 67 DIM (peak period). From de Souza and Lock (2017b).

## EFFECTS OF C16:0, C18:0, AND C/*S*-9 C18:1 ON PRODUCTION RESPONSES

We have recently carried out a series of studies examining the effect of individual saturated FA on production and metabolic responses of lactating cows. Piantoni et al. (2015a) reported that C18:0 increased DMI and yields of milk and milk components, with increases more evident in cows with higher milk yields, but the response occurred only in one of the two periods of the crossover design. Reasons why only higher yielding cows responded more positively to C18:0 supplementation and only in one period remains to be determined. Additionally, in a recent dose response study with mid lactation cows, feeding a C18:0-enriched supplement (93% C18:0) increased DMI but had no effect on the yields of milk or milk components when compared to a non-FA supplemented control diet, which was probably associated with the decrease in FA digestibility (Figure 1A, Boerman et al., 2017). Our results, and those of others, indicate that C16:0 supplementation has the potential to increase yields of ECM and milk fat as well as the conversion of feed to milk, independent of production level when it was included in the diet for soyhulls or C18:0 (Piantoni et al., 2013; Rico et al., 2014). We recently utilized a random regression model to analyze available individual cow data from 10 studies that fed C16:0-enriched supplements to post peak dairy cows (de Souza et al., 2016b). We observed that energy partitioning toward milk was increased linearly with C16:0 intake, as a result of a linear increase in milk fat yield and ECM with increasing intake of C16:0.

When we compared combinations of C16:0, C18:0, and *cis*-9 C18:1 in FA supplements, a supplement containing more C16:0 increased energy partitioning toward milk due to the greater milk fat yield response compared with the other treatments (de Souza et al., 2016a). In contrast, a FA supplement containing C16:0 and *cis*-9 C18:1 increased energy allocated to body reserves compared with other treatments. The FA supplement containing a combination of C16:0 and C18:0 reduced nutrient digestibility, which most likely explains the lower production responses observed compared with the other treatments. Interestingly, in a follow up study we compared different ratios of C16:0 and *cis*-9 C18:1 in FA supplements fed to post-peak cows, and observed that supplements with more C16:0 favored energy partitioning to milk in cows producing less than 45 kg/d, while supplements with more *cis*-9 C18:1 favored energy partitioning to milk in cows producing great than 60 kg/d (de Souza and Lock, 2017a). Also, regardless of production level, supplements with more *cis*-9 C18:1 increased BW change. This may suggest that C16:0 and *cis*-9 C18:1 are able to alter energy partitioning between the mammary gland and adipose tissue, which may allow for different FA supplements to be fed in specific situations according to the metabolic priority and needs of dairy cows. Further research is needed to confirm these results in cows at different stages of lactation or other physiological conditions.

In early lactation cows, Beam and Butler (1998) fed a saturated FA supplement (~ 40% C16:0 and 40% C18:0) and observed that FA supplementation decreased DMI and did not affect yields of milk and ECM in the first 4 weeks after calving. Piantoni et al. (2015b) fed a similar saturated FA supplement (~ 40% C16:0 and 40% C18:0) and observed that FA supplementation during the immediate postpartum (1-29 DIM) favored energy partitioning to body reserves rather than milk yield, especially in the lower forage diet. The high forage diet with supplemental FA increased DMI and tended to decrease BCS loss compared with the same diet without FA supplementation. Also, regardless of forage level, feeding supplemental FA increased DMI, decreased BCS loss, but tended to decrease milk yield. When cows were fed a common diet during the carryover period, the low forage diet with FA supplementation fed during the immediate postpartum continued to decrease milk yield and maintained higher BCS compared with the other treatments. On the other hand, Weiss and Pinos-Rodriguez (2009) fed a similar saturated FA supplement (~ 40% C16:0 and 40% C18:0) to early-lactation cows (21 to 126 DIM) and observed that when high-forage diets were supplemented with FA, the increased NE<sub>L</sub> intake went toward body energy reserves as measured by higher BCS with no change in milk yield. However, when low-forage diets were supplemented with FA, milk yield increased (2.6 kg/d) with no change in BCS.

In our recent study, we evaluated the effects of timing of C16:0 supplementation on performance of early lactation dairy cows (de Souza and Lock, 2017b). During the fresh period (1-24 DIM), we did not observe treatment differences for DMI or milk yield (Figure 5A), but compared with control, C16:0 increased the yield of ECM by 4.70 kg/d consistently over time (Figure 5B). However, C16:0 reduced body weight by 21 kg (Figure 5C), and body condition score by 0.09 units and tended to increase body weight loss by 0.76 kg/d compared with CON. Feeding C16:0 during the peak period (25 to 67

DIM) increased the yield of milk by 3.45 kg/d, ECM yield by 4.60 kg/d, and tended to reduce body weight by 10 kg compared with control (Figure 5).

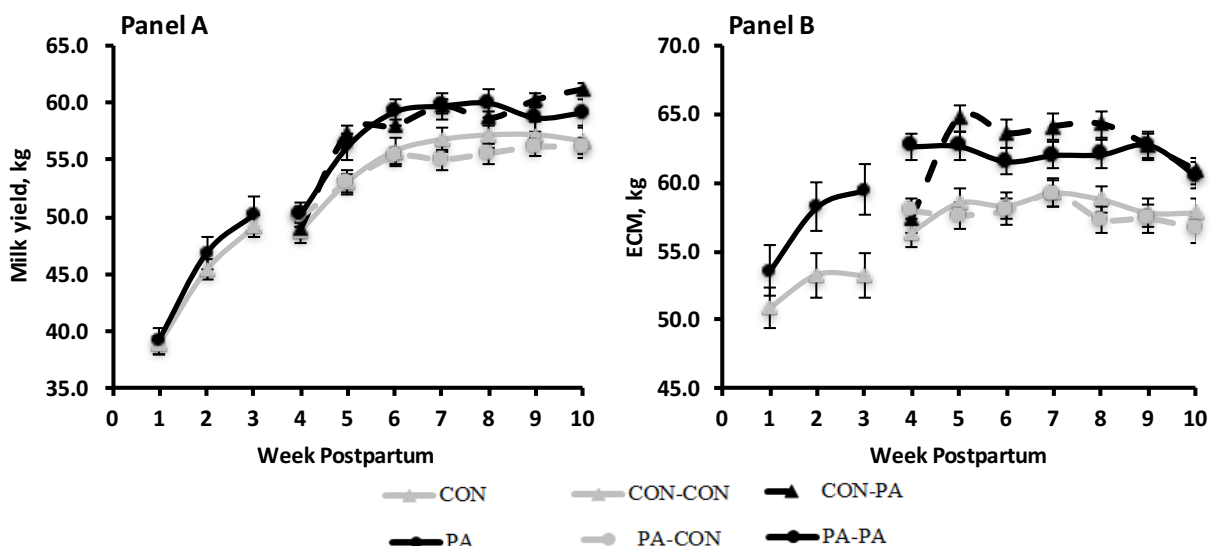


Figure 5. The effects of C16:0-enriched supplementation in early lactation cows on the yield of milk (Panel A) and ECM (Panel B). Results from 52 early-lactation cows receiving the following diets: no supplemental fat (CON) or a C16:0 supplemented diet (PA) that was fed either from calving (1 to 24 DIM; fresh period) or from 25 to 67 DIM (peak period). From de Souza and Lock (2017b).

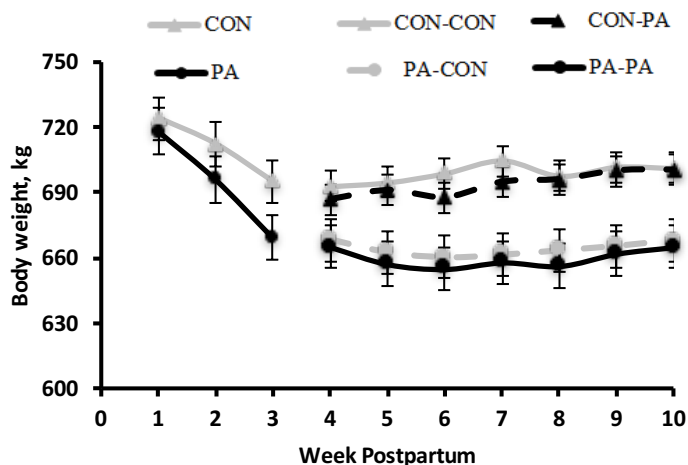


Figure 6. The effects of C16:0-enriched supplementation in early lactation cows on body weight. Results from 52 early-lactation cows receiving the following diets: no supplemental fat (CON) or a C16:0 supplemented diet (PA) that was fed either from calving (1 to 24 DIM; fresh period) or from 25 to 67 DIM (peak period). From de Souza and Lock (2017b).

Interestingly, Greco et al. (2015) observed that decreasing the ratio of omega-6 to omega-3 FA in the diet of lactating dairy cows while maintaining similar dietary concentrations of total FA improved productive performance in early lactation. A dietary omega-6 to omega-3 ratio of approximately 4:1 increased DMI and production of milk and milk components compared with a 6:1 ratio. Approximately 1.3 kg of milk response

could not be accounted for by differences in nutrient intake, which suggests that reducing the dietary FA ratio from 6:1 to 4:1 can influence nutrient partitioning to favor an increased proportion of the total net energy consumed allocated to milk synthesis. Further studies focusing on altering ratio of dietary FA are warranted, especially in early lactation cows.

## EFFECTS OF SUPPLEMENTAL FATTY ACIDS ON REPRODUCTION

A recent meta-analysis of 17 studies reported a 27% increase in pregnancy rate in the first postpartum artificial insemination (AI) when dairy cows were fed fat supplements during the transition period (Rodney et al., 2015). In addition, the interval from calving to pregnancy was reduced. The inclusion of the very long chain omega-3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the form of fish meal, fish oil, or algae in the diet has been shown to improve either first-service or overall pregnancy in 6 studies (Santos and Staples, 2017). A study conducted at the University of Florida (Silvestre et al., 2011) demonstrated that supplementation with Ca salts (1.5% of dietary DM) enriched in fish oil-derived FA starting at 30 DIM improved pregnancy rate/AI compared with Ca salts of palm FA (52.8 vs. 45.5%). Additionally, pregnancy loss between 32 and 60 d after AI was reduced by feeding Ca salts containing EPA and DHA (6.1 vs. 11.8%). Recently, Sinedino et al. (2017) observed that feeding 100 g/d of an algae product containing 10% of DM as DHA starting in the third week postpartum increased pregnancy rate by 39% and reduced days to pregnancy by 22 d (102 vs. 124 d). Therefore, polyunsaturated long-chain FA including omega-6 and omega-3 seem to be more effective at improving pregnancy in dairy cows than those containing mainly C16:0 and *cis*-9 C18:1. Furthermore, a meta-analysis indicated that the probability of pregnancy increased by 26% and the days from calving to pregnancy decreased by 34 d when *trans*-10, *cis*-12 conjugated linoleic acid was fed as a Ca-salt product across 5 studies involving 221 early lactation dairy cows (de Veth et al., 2009). Feeding long-chain FA might improve reproduction in dairy cattle through several potential mechanisms, including reducing negative energy balance, changes in follicle development and improvements in oocyte quality, improved early embryo development, and reduced pregnancy loss. Since individual FA have a direct effect on several metabolic processes, research should focus on determining “ideal” combinations of FA for cows under specific physiological conditions and for specific purposes.

## CONCLUSION

The addition of supplemental FA to diets is a common practice in dairy nutrition to increase dietary energy density and to support milk production. Although in general FA supplementation has been shown to increase milk yield, milk fat yield, and improve reproduction performance, great variation has been reported in production performance for different FA supplements, and indeed the same supplement across different diets and studies. Results are contradictory about the benefits of FA supplementation to early lactation dairy cows. We propose that this is a result of differences in FA profile of supplements used and the time at which FA supplementation starts. Further work is

required to characterize the sources of variation in response to FA supplementation. Just as we recognize that not all protein sources are the same it is important to remember that not all FA sources and FA supplements are the same. The key is to know what FA are present in the supplement, particularly FA chain length and their degree of unsaturation. Once this information is known it is important to consider the possible effects of these FA on DMI, rumen metabolism, small intestine digestibility, milk component synthesis in the mammary gland, energy partitioning between the mammary gland and other tissues, body condition, and their effects on immune and reproductive function. The extent of these simultaneous changes along with the goal of the nutritional strategy employed will ultimately determine the overall effect of the FA supplementation, and the associated decision regarding their inclusion in diets for lactating dairy cows.

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# DEVELOPING PRACTICAL APPROACHES TO MODIFY HEPATIC FATTY ACID PROCESSING AND LIPID MEDIATOR BIOSYNTHESIS IN DAIRY CATTLE: THE EMERGING ROLE OF LIPIDOMICS

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## INTRODUCTION

Postpartum metabolic disease in dairy cattle involves the development of adipose tissue insulin resistance and hepatic lipid accretion. Although the adverse interplay between these metabolic organs is accepted, the cellular mechanisms that contribute to impaired insulin action and steatosis are not completely understood. Indeed, early lactation insulin resistance promotes adipose tissue lipolysis and dyslipidemia increases the hepatic uptake of fatty acids (**FA**). As a consequence, steatosis develops because of inadequate mitochondrial  $\beta$ -oxidation, enhanced triacylglycerol (**TAG**) esterification, and the cow's limited ability to export TAG within very low density lipoproteins (**VLDL**). These hallmark metabolic features of the periparturient period increase a cow's risk of obtaining a postpartum metabolic disease, and can elicit long-term consequences including immunosuppression, compromised milk production, infertility, and reduced longevity. Therefore, it is imperative that we renew our commitment to innovate and develop practical approaches that improve peripartal health. To achieve this goal, we must employ a translational dairy science approach that focuses on characterizing the biochemical mechanisms of insulin resistance and fatty liver, while simultaneously developing practical nutritional strategies that are purposefully designed to target these mechanisms. A systems-biology approach that will help us achieve this goal is the application of mass spectrometry-based lipidomics.

The advent of lipidomics has revolutionized our ability to understand lipid metabolism within the context of metabolic disease (Puri et al., 2007; Holland and Summers, 2008). Certainly, we can acknowledge the immense complexity of the bovine lipidome (i.e. the complete lipid profile within a cell, tissue, or animal). To help manage our understanding of lipid metabolism, researchers have relied on the broad classification of lipids by their shared structural attributes (e.g. mono-/di-/triacylglycerol, sphingolipid ceramide, and glycerophospholipid phosphatidylcholine (**PC**)), and dairy science has been historically limited by the unavailability of technologies to study the bioactive diversity that exists within each lipid class. One exception is our advanced understanding of nonesterified FA. We can now appreciate that FA have unique functional properties. For instance, the abilities or inabilities of saturated or polyunsaturated FA (e.g. palmitic acid, *cis*-9, *cis*-12 linoleic acid, *trans*-10, *cis*-12 conjugated linoleic acid, or docosahexaenoic acid) to modify energy metabolism and influence health are routinely characterized (Kelsey et al., 2003; Stamey et al., 2012; Loften et al., 2014). The profiling and quantification of FA by gas chromatography coupled with single or tandem mass spectrometry is a classic example of targeted lipidomics. To quantify complex lipids with

structural specificity, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is frequently employed in biomedicine. Our lab has actively utilized targeted lipidomics with LC-MS/MS to quantify 150+ complex lipids including sphingolipids (ceramides, monohexosylceramide, lactosylceramide, and sphingomyelin), fatty acylcarnitines, and glycerophospholipids (PC and phosphatidylethanolamine (**PE**)) in bovine plasma, tissues (liver, skeletal muscle, and adipose), and/or isolated lipoproteins (e.g. Davis et al., 2017a; Rico et al., 2015a,b,c, 2016, 2017a,b; Phipps et al., 2017). We have also utilized an untargeted lipidomics approach involving cutting-edge time-of-flight mass spectrometry (TOF-MS) to biochemically map 1,400+ hepatic and plasma lipids spanning 18 lipid classes in periparturient dairy cows (e.g. Saed Samii et al., 2017). Most importantly, lipidomics is being utilized to characterize the mechanisms that mediate insulin resistance and fatty liver disease in dairy cattle. The advantage of using lipidomics is that the lipidome represents the downstream product of gene transcription and translation, therefore, the lipidome is closest to the metabolic phenotype. This review will highlight the integration and progression of lipidomics within the dairy sciences. Moreover, the proposed role of sphingolipid ceramide and glycerophospholipid PC within the framework of metabolic disease will be discussed (Figure 1).

## INSULIN RESISTANCE AND THE SPHINGOLIPID CERAMIDE

The mechanisms mediating insulin resistance in non-ruminants experiencing overnutrition involve the sphingolipid ceramide (Holland and Summers, 2008; Chavez and Summers, 2012). Due in part to limited mitochondrial FA oxidation, surplus saturated fatty acyl-CoA are diverted towards serine palmitoyltransferase and ceramide synthase within the *de novo* ceramide synthesis pathway. Alternatively, overnutrition and inflammation can promote ceramide accrual via the activation of acid sphingomyelinase. Acid sphingomyelinase controls the transformation of sphingomyelin into ceramide (Figure 1), and is activated by pro-inflammatory cytokines including tumor necrosis factor- $\alpha$ . The accumulation of ceramide in circulation, and skeletal muscle and adipose tissues represents a key biochemical feature of the pathophysiology that defines insulin resistance in overweight subjects experiencing lipotoxicity (Haus et al., 2009). To promote insulin resistance, ceramide can inhibit the activation of insulin receptor substrate 1 and protein kinase B to suppress insulin-stimulated glucose transporter translocation to the plasma membrane (Chavez and Summers, 2012). The inhibition of insulin signaling transduction by ceramide can trigger the protein kinase A-dependent activation of hormone sensitive lipase, thus increasing lipolysis (Mei et al., 2002). Although intracellular ceramide accumulation can promote insulin resistance, recent findings also suggest that liver-derived lipoprotein ceramide can also impair peripheral insulin action. Because hyperlipidemia, inflammation, steatosis, and insulin resistance are all linked with ceramide synthesis in non-ruminants, we are actively exploring the associative and causative roles for ceramide in dairy cattle.

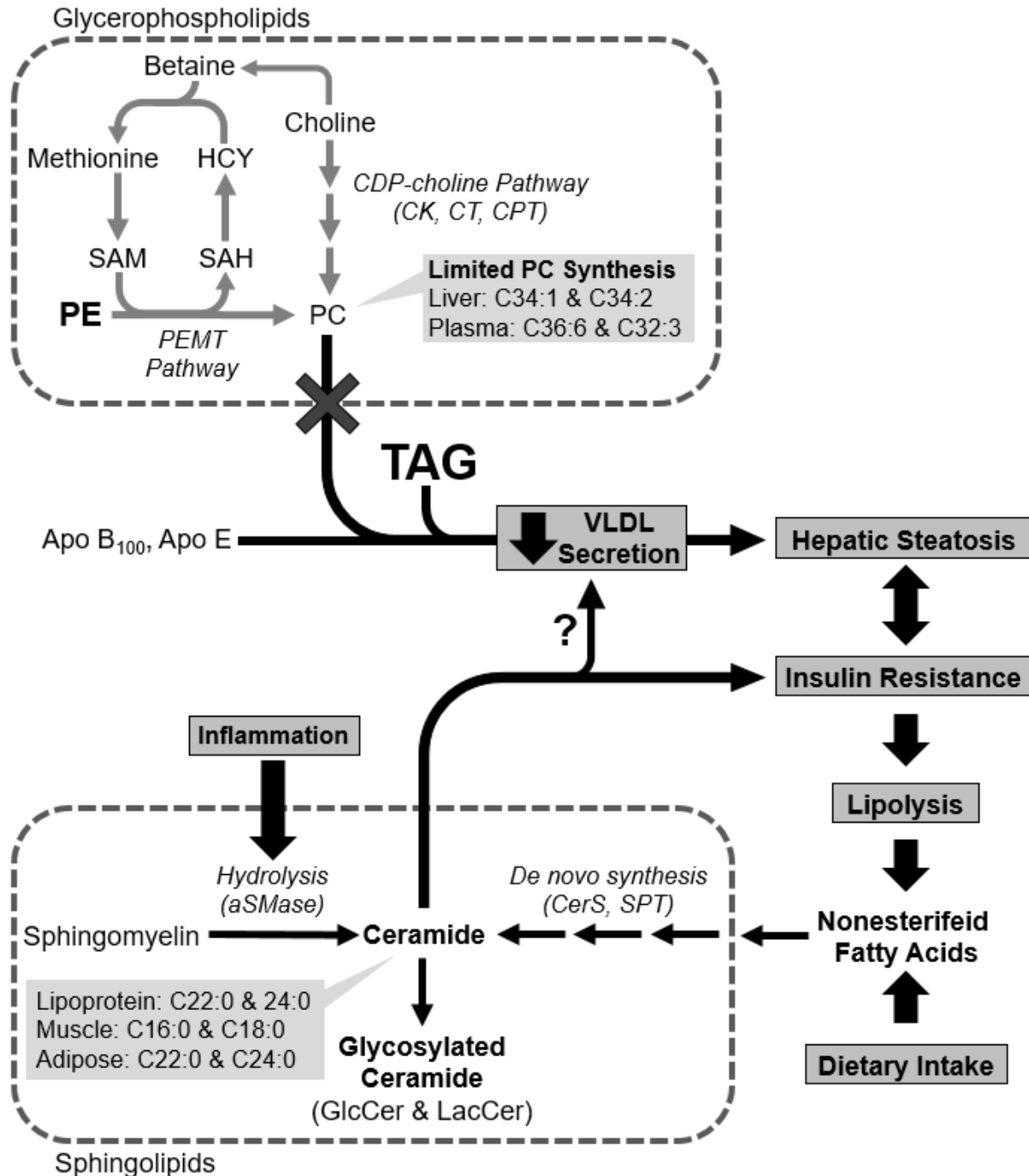


Figure 1. Proposed remodeling of the lipidome to promote insulin resistance and hepatic steatosis in the periparturient dairy cow. Metabolites and paths in bold reflect elevated levels or flux, respectively. aSMase, acid sphingomyelinase; CerS, ceramide synthase; CK, choline kinase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; CT, CTP:phosphocholine cytidyltransferase; GlcCer, monohexosylceramide; HCY, homocysteine; LacCer, lactosylceramide; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PC, phosphatidylcholine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SPT, serine palmitoyltransferase; TAG, triacylglycerol; VLDL, very low density lipoprotein.

We first demonstrated that the majority of circulating non-glycosylated and glycosylated ceramides increase in dairy cattle transitioning from gestation to lactation (Rico et al., 2015b, 2017a); however, the magnitude of increase is greater for cows with enhanced prepartum adiposity. Moreover, circulating ceramide is positively associated with the availability of nonesterified FA in plasma. These studies identified very long chain ceramides to be the most responsive (e.g. C24:0-ceramide), whereas, in a reciprocal manner, C16:0-ceramide selectively decreases postpartum. Recent data suggests that C24:0-ceramide can promote insulin resistance (Haus et al., 2009; Boon et al., 2013). In support, we demonstrated that plasma C24:0-ceramide is inversely associated with glucose clearance rates following an insulin challenge postpartum (Rico et al., 2017a); however, this relationship was not observed for circulating C16:0-ceramide. In contrast, skeletal muscle C18:0-ceramide was inversely associated with postpartum insulin-stimulated reductions of glucose. These findings suggest that the compartmentalization of specific ceramides may influence their ability to antagonize insulin action. One compartment of interest is liver tissue. Circulating ceramides are found predominantly within lipoproteins derived from liver, and lipoprotein C24:0-ceramide can inhibit insulin signaling in rodents (Boon et al., 2013). In our work, we have demonstrated that overweight cows with steatosis exhibit increased hepatic ceramide concentrations (Rico et al., 2017a), a response that develops in unison with circulating C24:0-ceramide supply. Others have demonstrated that enhanced hepatic FA uptake can increase de novo ceramide synthesis (Watt et al., 2012); however, we hypothesize that sphingomyelin hydrolysis may also contribute to ceramide synthesis in periparturient cows because they experience hepatic and systemic inflammation (Bradford et al., 2015). In support, we have demonstrated marked reductions in hepatic (unpublished) and circulating very long chain sphingomyelin concentrations (e.g. C24:0-sphingomyelin) during transition (Rico et al., 2017a), a response that develops with ceramide accrual. Although the determination of whether liver-derived lipoprotein ceramide mediates peripheral insulin action in cows is on-going, we recently developed a novel approach using fast protein liquid chromatography to isolate bovine lipoproteins for lipidomic evaluation (Phipps et al., 2017). We discovered that TAG-rich lipoproteins (e.g. VLDL) are enriched in C24:0-ceramide. The current goal is to characterize changes in lipoprotein ceramide in relation to peripartal insulin sensitivity, and determine whether lipoprotein ceramide causes adipocyte insulin resistance and lipolysis.

To further characterize ceramide metabolism in cows, we employed two in vivo models to induce hyperlipidemia in nonlactating, nongestating dairy cows. First, dairy cows were intravenously infused with a TAG emulsion for 16 h (Rico et al., 2015c). Second, dairy cows were nutrient-restricted for 32 h (Davis et al., 2017a). In both experiments, circulating FA and hepatic lipid deposition were enhanced by hyperlipidemia-induction. Similar to the transition cow, we observed marked elevations in circulating and hepatic ceramide concentrations. Again, C24:0-ceramide was most responsive. In TAG-infused cows, we did not observe a change in circulating sphingomyelin levels. These data suggest that de novo ceramide synthesis is the preferred route for ceramide synthesis in healthy cows. In support, we observed a significant increase in hepatic ceramide synthase 2 mRNA expression (controls C24:0-

ceramide synthesis; unpublished). In nutrient-restricted cows, only C22:0- and C24:0-ceramide were inversely related to insulin-stimulated glucose uptake.

If ceramide mediates insulin resistance in dairy cows, then we must be cognizant of diets that promote ceramide synthesis. Therefore, we focused our attention on palmitic acid feeding. Palmitic acid is substrate for serine palmitoyltransferase within the de novo ceramide synthesis pathway. First, we evaluated palmitic acid feeding in mid-lactation dairy cows versus no added fat control cows (Mathews et al., 2016; Rico et al., 2016). Palmitic acid feeding rapidly increased circulating ceramide, especially C24:0-ceramide. Additionally, palmitic acid feeding increased hepatic ceramide concentrations, and ceramides were inversely related to FA disappearance following a glucose challenge. We were also surprised to observe a gradual reduction in plasma ceramide levels as control cows advanced through lactation (138 to 201 DIM), a response that was decelerated by palmitic acid feeding. This gradual decline in ceramide concentrations developed with a progressive decrease in circulating FA and a gradual rise in plasma insulin. Later, we confirmed that palmitic acid feeding promotes ceramide accumulation in early lactation cows (versus no added fat controls; Davis et al., 2017b), and palmitic acid feeding is more effective at increasing ceramide synthesis than stearic acid feeding (Rico et al., 2017b). Most notably, across all three studies, circulating ceramide was positively correlated with milk yield. Our targeted lipidomic data suggest that ceramides may be intrinsically involved in the homeorhetic adaptation to lactation.

It is clear that ceramide is a biomarker for impaired insulin action in dairy cattle, particularly C24:0-ceramide. Developing nutritional strategies that target C24:0-ceramide synthesis has the potential to control insulin action in dairy cattle. First, decreasing ceramide synthesis during early lactation may be a means to improve insulin sensitivity and inhibit adipose tissue lipolysis. Such an approach would reduce hepatic FA uptake and minimize the prevalence of steatosis; however, we recognize the possibility that ceramide may be a fundamental promoter of nutrient partitioning and lactation. Therefore, decreasing ceramide synthesis may only be best served for cows at elevated risk for metabolic disease (e.g. cows with elevated prepartum adiposity). Second, increasing ceramide synthesis beyond peak milk production may be a means of inducing a homeorhetic response to maximize milk production. Moving forward, we need to continue to evaluate the role of dietary FA as modifiers of ceramide supply. For instance, polyunsaturated FA may not induce de novo ceramide synthesis because the pathway preferentially utilizes saturated FA substrate. We are actively testing this hypothesis using lipidomics. Additionally, our team is exploring novel nutritional approaches to specifically target the synthesis of C24:0-ceramide in mid-lactation dairy cows.

## FATTY LIVER AND THE GLYCEROPHOSPHOLIPID PHOSPHATIDYLCHOLINE

Nonesterified FA are activated to fatty acyl-CoA; however, the catabolic or anabolic processing of fatty acyl-CoA in liver is influenced by the enhanced rate of FA uptake during the transition period. First, the capacity to completely oxidize palmitate to CO<sub>2</sub> is not enhanced during the transition from gestation to lactation (Litherland et al., 2011; McCarthy et al., 2015). In contrast, incomplete oxidation to acid-soluble products (i.e. TCA

cycle intermediates or ketones) is maximum during peak lipolytic response (Dann et al., 2006; Litherland et al., 2011). In non-ruminants with steatosis, hepatic FA influx develops with the accumulation of acylcarnitines in circulation (e.g., palmitoylcarnitine; Chen et al., 2016). Acylcarnitines are intermediates involved in the mitochondrial uptake of fatty acyl-CoA and represent biomarkers for defective hepatic mitochondrial  $\beta$ -oxidation (Adams et al., 2009; Schooneman et al., 2013). In support, our targeted lipidomic analyses have demonstrated increased plasma concentrations of acylcarnitines in transition cows (Rico et al., 2015a). As a consequence of increased hepatic FA uptake and inadequate oxidation, fatty acyl-CoA are partitioned towards TAG esterification which is mediated in part by the activation of acyltransferases.

A major contributing factor of postpartum steatosis is the cow's reduced capacity to export VLDL TAG from liver, relative to non-ruminants (Pullen et al., 1990). Our untargeted lipidomic approach has confirmed dramatic reductions in circulating TAG ranging from 18 to 97% (mean of 77%; d -28 to nadir d 3 postpartum) in 49 unique circulating TAG during the transition from gestation to lactation (e.g., TAG 60:1, 62:0, 56:1, 62:1, 58:1, and 56:4; Saed Samii et al., 2017). In parallel with a decrease in plasma TAG, we have observed similar reductions in 64 plasma monoalkyl-diacylglycerols (MADAG) during the peripartum (e.g. MADAG 52:1, 58:0, and 54:5). MADAG are ether-linked neutral lipids that aggregate with TAG and cholesteryl esters within hepatic lipid droplets and may be involved in ether phospholipid formation (Bartz et al., 2007). Our results suggest that MADAG may be transported from liver with TAG; however, the importance of MADAG for VLDL secretion is unknown. One possible explanation for limited VLDL export in cows is limited hepatic apolipoprotein (**Apo**) B<sub>100</sub> concentrations. Apo B<sub>100</sub> is required for VLDL assembly and secretion; however, the expression of hepatic Apo B<sub>100</sub> mRNA as well as circulating Apo B<sub>100</sub> concentrations decrease as parturition approaches, and are inversely related to FA levels (Bernabucci et al., 2004; Bernabucci et al., 2009).

The most promising explanation for impaired VLDL TAG export is limited supply of hepatic PC, as proposed by Van den Top et al. (1996). Glycerophospholipids form a monolayer on the lipoprotein surface surrounding the hydrophobic core, and PC is the most abundant glycerophospholipid component in all lipoprotein subclasses. For example, PC comprises ~70% (mol %) of total phospholipids of rodent plasma VLDL, whereas lyso-PC (3%) and PE (4%) are representative of minor glycerophospholipid components (Ågren et al., 2005). Furthermore, reduced levels of hepatic PC impair the secretion of VLDL from the liver (Verkade et al., 1993; Fast and Vance, 1995). Although undefined in dairy cattle, the strict requirement of PC for VLDL secretion has been demonstrated in rat hepatocytes and CTP:phosphocholine cytidylyl-transferase deficient mice (Yao and Vance, 1988; Jacobs et al., 2004). Currently, no additional evidence exists for any other glycerophospholipid requirement for VLDL secretion including PE, phosphatidylserine, phosphatidylglycerol, or the corresponding lyso-phospholipids. However, PE may be required for VLDL assembly considering that nascent VLDL contain four times more PE than plasma VLDL (Hamilton and Felding, 1989). Although little is known about the contribution of PC to VLDL synthesis and secretion in cattle, we have established that circulating total PC reaches a nadir at parturition (Saed Samii et al.,



2017), and the majority of hepatic PC decrease during the transition from gestation to lactation (e.g., PC 31:1, 37:3, and 39:5; ~60% of 118 profiled; unpublished data). Moreover, we identified multiple PC that are suppressed and predictive of postpartum fatty liver disease (e.g. PC 36:6, 32:3, 34:4, 32:2, and PC 34:6; Saed Samii et al., 2017). However, we noticed that not all circulating PC species are positively correlated with plasma TAG concentrations (e.g., PC 38:2, 42:2). Additionally, counter to common belief, the levels of a select number of hepatic PC increase postpartum (e.g., PC 34:1, 34:2, 36:2; ~25% of 118 profiled). These lipidomic data highlight the complexity of the glycerophospholipidome. Moving forward, the generalization of glycerophospholipid classes must be avoided, and the diverse structure and function of PC should be emphasized. If we can identify specific PC (i.e. acyl moieties) that are most critical for VLDL assembly and secretion, we can then develop novel nutritional approaches to target these unique PC.

The synthesis of PC occurs via two independent pathways. First, the CDP-choline pathway (i.e. Kennedy pathway) requires choline and involves three enzymatic reactions controlled by choline kinase, CTP:phosphocholine cytidyltransferase, and CDP-choline:1,2-diacylglycerol cholinephosphotransferase. Alternatively, the phosphatidylethanolamine *N*-methyltransferase (**PEMT**) pathway involves three sequential methylations of PE, and is quantitatively relevant in liver tissue where it contributes approximately 30% of total hepatic PC synthesis (Sundler and Akesson et al., 1975). The activation of PEMT is highly dependent on the transmethylation cycle, a metabolic pathway which produces the prerequisite methyl donor *S*-adenosylmethionine (**SAM**). In liver, where PC demand for VLDL assembly and secretion is high, SAM homeostasis ensures the methylation of PE to PC (Lu, 2000). To maintain PE methylation, the transmethylation cycle can be activated by methionine and betaine (trimethylglycine) which are important dietary sources of labile methyl groups. Additionally, the irreversible oxidation of choline by choline dehydrogenase and betaine aldehyde dehydrogenase generates betaine. The availability of hepatic methionine and betaine for PC synthesis can be limited during the transition from gestation to lactation (Zeisel et al., 1995); however, evidence in rodents has demonstrated that increasing hepatic supply of methionine and betaine can increase PC synthesis and VLDL secretion (Sugiyama et al., 1998; Kharbanda et al., 2007). Because increasing hepatic PC synthesis may be a means to increase bovine VLDL synthesis and export during the periparturient period, one dietary approach to increase hepatic PC synthesis is methyl donor supplementation.

Considering that choline is the precursor for the CDP-choline pathway, and choline, methionine, and betaine are all potential methyl donors, research focused on the periparturient supplementation of these nutrients has been frequent (Piepenbrink and Overton, 2003; Davidson et al., 2008; Grummer, 2008). Initial studies with rumen-protected (**RP**) choline supplementation (variable doses) during the peripartum found no effects of choline supplementation on liver TAG concentrations (Hartwell et al., 2000; Piepenbrink and Overton, 2003; Zahra et al., 2006); albeit, Zom et al. (2011) later demonstrated the hepatic TAG-lowering ability of RP choline (~15 g/d of choline) in dairy cows. Transition cows supplemented with 15 g/d of RP methionine exhibit lower plasma

FA and  $\beta$ -hydroxybutyrate levels, while displaying elevated circulating VLDL and Apo B<sub>100</sub> concentrations (Sun et al., 2016); although these responses may be due in part to enhanced dry matter intake. Recent evidence has demonstrated the ability of RP methionine to increase methionine adenosyltransferase 1A mRNA expression within the transmethylation cycle (Osorio et al., 2014). Research investigating betaine supplementation in dairy cows is limited. Supplementing a corn-silage total mixed ration (formulated to contain limited Met) with RP betaine (45 g/d) did not improve metabolic health or milk production (Davidson et al., 2008). In contrast, supplemental anhydrous betaine ( $\geq 50$  g/d) lowered plasma FA and  $\beta$ -hydroxybutyrate concentrations, and increased milk yield in mid-lactation dairy cows (Wang et al., 2010). Unfortunately, the measurement of hepatic or VLDL PC in response to methyl donor supplementation has historically not been evaluated. In an initial investigation, we utilized lipidomics to demonstrate the ability of methyl donor supplementation (100 g/d; containing 22 g/d methionine, 10 g/d of choline chloride, and 3 g/d of betaine; MecoVit®; Vetagro S.p.A.; Zang et al., 2017) to increase multiple circulating TAG in periparturient dairy cows (e.g. TAG 46:2 and 46:3; Zang et al., 2017). We are actively evaluating the efficacy of methyl donors to increase hepatic PC synthesis and VLDL export using a combination of fast protein liquid chromatography for lipoprotein isolation and targeted lipidomics for complete PC quantitation.

#### POTENTIAL INTERPLAY BETWEEN CERAMIDE AND PHOSPHATIDYLCHOLINE

In the context of hepatic steatosis, the potential interplay between sphingolipid and glycerophospholipid synthesis in liver deserves attention because recent data suggests that ceramide can antagonize hepatic PC synthesis. Specifically, ceramide can inhibit CTP:phosphocholine cytidyltransferase and CDP-choline:1,2-diacylglycerol cholinephosphotransferase within the CDP-choline pathway (Bladergroen et al., 1999; Ramos et al., 2002), and inhibit hepatic methionine adenosyltransferase gene expression and activity within the transmethylation cycle (Frago et al., 2001). Because we have demonstrated increased circulating and hepatic ceramide supply in overweight cows that develop steatosis (Rico et al., 2015b, 2017a), and hepatic PC levels are significantly suppressed in postpartum cows, the potential ability of ceramide to inhibit PC synthesis in the periparturient cow should be explored. If confirmed, then ceramide accrual may negate the efficacy of methyl donors to increase PC synthesis, and dietary approaches that decrease ceramide synthesis may be a means to maximize methyl donor efficacy. These uncertainties will need to be addressed moving forward.

#### CONCLUSION

The use of mass spectrometry-based lipidomics has emerged as a powerful analytical tool to understand dairy cattle nutrition and metabolism. The discovery of the sphingolipid ceramide and glycerophospholipid phosphatidylcholine as respective biomarkers of insulin sensitivity and hepatic steatosis in dairy cattle represents an early effort to utilize lipidomics. Because lipidomics can generate large data sets, the refinement of bioinformatic approaches to analyze and facilitate data interpretation represents an academic and industry challenge. This challenge can be in part overcome

by adhering to hypothesis-driven research. Lastly, the continued application of lipidomics and development of nutrition-based approaches has the potential to improve cow health; however, metabolic disease lipid mediators should be actively targeted.

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## THE AMINO ACID CONTENT OF RUMEN MICROBES, FEED, MILK AND TISSUE AFTER MULTIPLE HYDROLYSIS TIMES AND IMPLICATIONS FOR THE CNCPS

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Nutrient supply and requirement models such as the Cornell Net Carbohydrate and Protein System (**CNCPS**) (Higgs et al., 2015; Van Amburgh et al., 2015) and the NRC (NRC, 2001) predict post-ruminal flows of nutrients from feed proteins that escape rumen fermentation and rumen microbes. Feed and microbial protein flow has an AA content, AA profile, and a digestibility of individual AA to calculate supply of metabolizable AA. The CNCPS has been updated into a new dynamic framework (v. 7; Higgs, 2014; Higgs et al. submitted) where all amino acids and other protein components are described by their nitrogen (N) content and the model uses a more mechanistic approach than previous versions as it accounts for protozoa and endogenous AA contributions to total AA flow in addition to bacteria and feed. To improve this model, accurate representations of the AA content and digestibility of all sources of AA were needed to understand sources of error in predictions of particular AA. On an N basis, the predictions of non-ammonia nitrogen (NAN) in CNCPS v. 7 were reasonably accurate and precise. However, predictions of individual AA such as Lys, Met, Ile, Leu, and Val were biased, suggesting there was a lack of information about the true content those AA in both microbes and feeds and possibly their digestibility (Fessenden et al., 2017; Higgs, 2014; Higgs et al., submitted).

The CNCPS feed library was recently updated with new chemical composition information, especially the AA profile and content of feeds (Higgs et al., 2015). Further, the CNCPS uses the AA profile of bacteria obtained from the literature (Storm et al., 1983; Clark et al., 1992; Volden and Harstad, 1998), and few of those studies accounted for protozoal AA flows, which can contribute a substantial amount to total microbial AA flow (Dijkstra et al., 1998; Fessenden, 2016). Given the updated AA information in the feed library, the biased flow predictions of certain AA in the CNCPS v7 was surprising because the AA are described on a N basis and there was no bias in the prediction of NAN, thus to observe bias in the predictions of the AA when the total NAN was decomposed into individual AA suggests two possibilities: the AA profiles and contents of feed and microbes are not correct and or the digestibility of the fractions are misunderstood or unknown. Therefore, we needed to challenge the information that was available to us describing both AA content and digestibility of both rumen microbes and feed.

The AA content of feeds and microbes have historically been determined by single time point hydrolysis, as this represents a compromise between maximal release of AA from the matrix while minimizing the loss of acid labile AA (Rutherford, 2009). Determination of AA at multiple time points followed by least-squares non-linear regression appears to provide more accurate estimates of the AA profile (Darragh and Moughan, 2005). This approach has been utilized in purified protein (Darragh et al., 1996), milk protein (Rutherford et al., 2008) and common animal feedstuffs (Rutherford,



2009). Previous work in our laboratory indicated that to obtain the greatest release of branched-chain AA in forages, hydrolysis times needed to be greater than 21 hr and Ile release was greatest at 70 hr (Ross, 2004) but we did not challenge the literature at that time. It was apparent that as the hydrolysis times were extended, certain AA were destroyed, whereas others demonstrated greater release from the carbohydrate matrix which suggested the use of multiple time point hydrolysis to observe optimum recovery of all AA.

Given the data from Darragh and Moughan (2005) and Rutherford (2009) and the observations made from the data of Higgs et al. (submitted), the hypothesis of this work was that the standard method of determination of AA in feed, milk, tissue and ruminal bacteria and protozoa using single time point hydrolysis is not equivalent to AA determination after multiple time point hydrolysis and non-linear least-squares regression. The implications for model libraries, efficiencies of use and AA formulation is significant and requires further evaluation as we move closer to more precise and accurate predictions of AA requirements and supply.

## Materials and Methods

Bacteria and protozoa included in the analysis were from the following experiments: Trial A: An omasal sampling trial with 8 cows in a 2 treatment switchback design investigating effects of a commercial byproduct feed on omasal nutrient flow (Fessenden, 2016); Trial B: An omasal sampling trial with 12 cows in a 3 treatment Latin Square design investigating the effect of rapidly degradable starch on omasal nutrient flow (Foskolos et al., unpublished data); Trial C: A ruminal N balance and recycling trial with 12 cows in a 3 treatment randomized complete block design investigating ruminal N and/or MP deficient diets (Recktenwald, 2010; Recktenwald et al., 2013). One additional protozoal sample was obtained from T. Hackmann at the University of Florida from repeated isolations from the rumen of a lactating dairy cow at the Ohio State University Columbus campus (Trial D). For trials A-C, equal parts DM were combined within microbial type, resulting in a composited sample of bacteria and protozoa from each experiment. Due to limited amount of sample for some trials (D and C) not all analysis were performed on all samples as noted throughout the text.

Bacterial isolations for trial A and C were performed according to Whitehouse et al. (1994) with modifications. Briefly; whole omasal contents were filtered through 4 layers of cheesecloth and solids were rinsed once with saline, and the filtrate (I) was treated with formalin (0.1% v/v in final solution) and stored at 4°C. The solids retained on the cheesecloth were incubated for 1 h at 39 °C in a 0.1% methylcellulose solution, mixed for 1 min at low speed (Omni Mixer, Omni International, Kennesaw, GA) to detach solids associated bacteria, and held at 4°C for 24 h. The contents were then squeezed through 4 layers of cheesecloth and the filtrate (II) was treated with formalin (0.1% v/v in final solution). Filtrates I and II were then combined and centrifuged at 1,000 x g for 5 min at 4 °C to remove small feed particles and protozoa. The supernatant was centrifuged at 15,000 x g for 20 min at 4 °C and the bacterial pellet, representing both solid and liquid associated bacteria, was collected and stored at -20 °C until lyophilization and later

analysis. Bacterial isolation for trial B followed the same procedure as described above, however formalin was not used. Protozoa from trials A and B were isolated from whole contents using the same procedure as described by Denton et al. (2015) and modified as reported in Fessenden et al. (2017). The only difference between trials was the omission of formalin and centrifugation in Trial B.

Twenty-six feed samples, which were previously used in studies evaluating omasal flow of nutrients from lactating dairy cattle were chosen for this study, as well as six tissue samples that were collected from past experiments in the laboratory (Diaz et al., 2001; Meyer, 2005) and four milk samples that varied in MUN collected over three days. The feed samples were previously analyzed for complete chemical analysis and used in the CNCPS (v7, Higgs, 2014) to formulate diets for experiments in which AA content of the diets were known and the flow of such nutrients from the rumen was measured in the experiments.

Feed, tissue and milk samples were analyzed for dry matter (DM) after 16 h at 105°C (AOAC, 2016). Milk samples were analyzed for dry matter after freeze drying for 24h. Total feed and tissue N was determined using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St. Joseph, MI). The AA content of all feed samples was determined by HPLC following hydrolysis at 110°C in a block heater (Gehrke et al., 1985) for 2, 4, 6, 12, 18, 21, 24, 30, 48, 72, 120 and 168 h. For tissues and milk only 21, 72 and 168 h time points were used. The time points chosen were based on publications by Rutherford et al. (2008) and Rutherford (2009) where long-term hydrolysis was shown to release certain AA from the sample, thus increasing the estimated AA content of various substrates that were used in each individual study. In addition, the time points were similar to a previous study performed in our laboratory on rumen and omasal microbial composition, which demonstrated similar AA outcomes as hydrolysis time was extended (Fessenden et al., 2017). For Trp in tissue and milk, only 16 h and 24 h time points were used as it has been shown that there is no significant change in Trp concentration after these time points. The entire time course was performed twice for each sample using acid-washed (50% nitric acid) glassware, and the reported values are the mean of the two determinations.

### Standard Acid Hydrolysis

Samples containing 2 mg of N was weighed into Teflon-lined screw top hydrolysis tubes and 50 µL of 125 mM norleucine was added as an internal standard, as well as 5 mL of high-purity 6 M HCl. The tubes were then flushed with N<sub>2</sub> gas for 10 seconds and placed in boiling water for 10 minutes to remove oxygen. The samples were hydrolyzed as described above for the different time points. After hydrolysis, the tubes were cooled slightly and the tube contents were filtered through Whatman 541 filter paper and the filtrate was diluted to 50 mL in a volumetric flask with HPLC grade H<sub>2</sub>O. Aliquots of 0.3 mL were frozen at -20°C to prevent loss of various AA, such as Ser, Thr and Tyr (Gehrke et al., 1985). Aliquots were evaporated at 65°C under constant N<sub>2</sub> flushing, with 3 rinses and re-evaporations with HPLC grade H<sub>2</sub>O to remove acid residues, as indicated by the smell of chlorine. After final evaporation, the hydrolysate was dissolved in 0.6 mL of Na

diluent and analyzed by the HPLC (Na220, Pickering Laboratories, Mountain View, CA).

### Sulfur AA Acid Hydrolysis

Analyzing the sulfur-containing AA, Met and Cys, samples containing 2 mg of N and the internal standard, norleucine, were pre-oxidized with 1 mL performic acid to be analyzed as cysteic acid and methionine sulfone. Performic acid was prepared by combining 0.9 mL of 88 % formic acid, 0.1 mL of 30% H<sub>2</sub>O<sub>2</sub> and 5 mg phenol, incubated for 40 minutes at room temperature and constant stirring, and moved to an ice bath at 4°C for 20 minutes. The tubes were then sonicated in slushy water for 15 minutes and transferred to an ice bath at 4°C for 16 h (Mason et al., 1980). The oxidizing reaction was stopped and the excess performic acid reduced by adding 0.2 mL of concentrated HCl and allowing the tubes to stand for 15 minutes. For tubes being used for the 120h and 168h time points, the tubes were placed under vacuum using a water aspirator to remove residual PA and HCl (Elkin and Griffith, 1985). All tubes were then hydrolyzed and filtered as described above for standard acid hydrolysis.

### AA Analysis by HPLC

Individual AA hydrolysates were separated using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) fitted with a sodium cation exchange column (Cat. no 1154110T, Pickering Laboratories, Mountain View, CA) using a 4-buffer step gradient and column temperature gradient. Detection of separated AA was performed at 560 nm following post-column ninhydrin derivation. Standards (250 nM/mL) for the individual AA were prepared by diluting a pure standard in sample buffer. The volume of sample and standards loaded onto the column was 10 µL.

### Tryptophan Hydrolysis and Analysis

For Trp determination, a separate aliquot of sample containing 2 mg N was added to a Teflon-lined screw top hydrolysis tube with 1.2 g of Ba(OH)<sub>2</sub>, 0.125 ml of 5-methyl-trp (5MT), and about two mL of HPLC grade H<sub>2</sub>O. The tubes were flushed with N<sub>2</sub> gas for 10 seconds and placed in boiling water for 15 minutes to remove oxygen. The tubes were hydrolyzed at 110°C for the same time course as used for the other AA on a block heater (Landry and Delhaye, 1992; Ross, 2004). Included in the hydrolysis was 125µL of 5-Methyl- Trp (4mM) as an internal standard. Tubes were removed quickly and the contents were transferred to an Eppendorf tube and placed in ice. After cooling to precipitate barium ions, an aliquot (3 µL) of the hydrolysate was added to 1 mL of acetate buffer (0.07 M sodium acetate) and analyzed by HPLC in which AA were detected by fluorescence (excitation = 285 nm, emission = 345 nm).

## Calculations and Statistical Analysis

The AA concentrations were corrected for norleucine, the internal standard, using Equation 1 and calculated as mg AA g DM<sup>-1</sup> with Equation 2 (Ross, 2004).

Equation 1. Norleucine correction

$$\text{Corrected } nM \frac{AA}{ml} = \frac{\left( nM \frac{AA}{ml} \text{ from chromatogram} \right) \times \left( nM \frac{Norl}{ml} \text{ added} \right)}{\left( nM \text{ Norl from chromatogram} \right)}$$

Equation 2. Amino acid content of residue, mg AA g DM<sup>-1</sup>

$$\text{mg AA g DM}^{-1} = \frac{\left( \text{Corrected } nM \frac{AA}{ml} \times AA \text{ MW} \times \text{hydrolyzed sample volume, ml} \right)}{\left( \text{sample wt, g} \times 10^6 \times \text{residue DM, } \frac{g}{g} \right)}$$

Determination of the true AA concentration of feed was performed using a method similar to that of Fessenden et al. (In press, 2017). Each AA concentration (mg/g of DM) was plotted against hydrolysis time using the following a non-linear equation:

$$B(t) = \frac{A_0 h (e^{-lt} - e^{-ht})}{h - l}$$

where  $B(t)$  is the AA concentration at time  $t$ ,  $h$  is the hydrolysis rate (proportion of bound AA hydrolyzed per hour),  $l$  is the loss rate (proportion of bound AA destroyed per hour) and  $A_0$  is the actual AA content of the protein within the sample (Rutherford 2008; Rutherford et al. (2009). The variables,  $A_0$ ,  $h$  and  $l$  for each sample were derived from each AA using least-squares non-linear regression with the constraints that  $A_0 > 0$ , and  $h > 0$ , using SAS version 9.4 (SAS institute, Cary, NC). The 24h (21h) and 168h AA concentrations were compared for each EAA and feed, milk, or tissue and a T-test was performed to measure significance between concentrations. Significance was declared when  $P < 0.05$  and a trend was identified at  $P < 0.10$ .

## Microbial AA Content and Profiles

The release of individual AA in trial B bacteria and protozoa are in Tables 1 and 2. Extraction of Ile, Met, and Val demonstrated greater release over time and thus positive slopes at time points greater than 24 h and hydrolysis rate were lowest for these AA. Of the NEAA of the protozoa, Ala, Cys and Pro demonstrated increasing concentrations of AA as hydrolysis time increased. Overall, total AA were hydrolyzed from the sample matrix at a rate of 0.415 and 0.357 mg/h for bacteria and protozoa, respectively. The same least-squares non-linear regression approach has been previously employed in the analysis of other AA containing compounds, including lysozyme (Darragh et al., 1996), cat hair (Hendriks et al., 1998), human milk (Darragh and Moughan, 1998) and some common feedstuffs (Rutherford, 2009). Rutherford (2009) reported similarly low  $h$  for Ile and Val, while Ser was reported to have the highest  $l$  of any AA.

The use of multiple hydrolysis times provides some insight into the appropriateness of single time point hydrolysis for AA in rumen microbial samples. While both techniques are simply estimates of the theoretical unknown true AA composition, the regression method has been shown to more accurately estimate the true AA profile in purified proteins (Darragh et al., 1996). The AA profile determined from the regression compared with the value determined at 24 h was used to establish the equivalency of the two methods in relation to biologically relevant ranges (Table 6). This alternative framework of hypothesis testing requires thoughtful interpretation of the results. While some AA may exhibit negligible mean differences between analysis method, such as His and Thr, the interpretation of the 90% CI indicates that they are not equivalent, as the CI lies outside the pre-determined range of biologically relevant differences. Of the bacterial AA, the 24 h time point method was determined to be not equivalent to the multiple time point hydrolysis method for every AA except Gly. The 90 % CI of the mean difference was greater than  $\pm 1$  g/100g AA for Ile, Leu, Met, and Val. The relatively large underestimation of Ile, Met, and Val results in an overestimation of approximately 5% for the rapidly hydrolyzed AA such as Arg, Leu, and Lys. This is similar to the results of Rutherford (2009), where soybean meal Ile content was underestimated by 8.4 %, followed by Val (7.0%), Ser (4.6%), and Thr (4.3%). The relatively low range in acceptable equivalence (mean difference of -0.4 to 0.4 g/g100 AA for bacteria) serves to emphasize the importance of the AA profile of bacteria on AA supply determinations.

Table 1. Comparison of the AA composition (g/100 g AA) of omasal bacteria from trial B<sup>1</sup> determined using multiple hydrolysis time point or single hydrolysis time point methods (Fessenden et al., 2017).

AA	Method		S - M	SED <sup>2</sup>	90 % CI		EQ <sup>3</sup>
	Single	Multiple			Lower	Upper	
Essential AA							
Arg	5.00	4.73	0.27	0.03	0.06	0.48	No
His	2.12	2.11	0.01	0.14	-0.85	0.86	No
Ile	4.05	4.62	-0.58	0.46	-3.46	2.31	No
Leu	5.60	5.32	0.28	0.26	-1.35	1.91	No
Lys	7.54	7.17	0.37	0.04	0.11	0.63	No
Met	4.49	4.63	-0.14	0.36	-2.41	2.13	No
Phe	6.00	5.77	0.23	0.09	-0.31	0.77	No
Thr	5.49	5.53	-0.04	0.10	-0.69	0.60	No
Trp	5.97	5.77	0.20	0.03	-0.01	0.41	No
Val	5.92	6.32	-0.41	0.32	-2.41	1.60	No

<sup>1</sup>Trial B: Foskolos et al., (unpublished data). n=2 for all comparisons.

<sup>2</sup>Standard error of the difference.

<sup>3</sup>Equivalence determined from 2 one-sided paired t-tests. Methods deemed to be equivalent if 90% CI falls within defined equivalency of -0.4 to 0.4 g/100g of AA.

Protozoa AA determinations between methods showed more general agreement between hydrolysis methods, largely due to the greater range in equivalence limits (mean difference of -1.5 to 1.5 g/100g AA for protozoa). Six of the 10 EAA were deemed equivalent between methods (Table 2). Similar to the bacterial results, Ile and Met were

underestimated (13.4 and 16.5 %, respectively) when determined with a single time point hydrolysis, resulting in over estimation of several other AA, namely Lys.

Table 2. Comparison of the AA composition (g/100 g AA) of omasal protozoa from trial B<sup>1</sup> determined using multiple vs. single time point hydrolysis methods (Fessenden et al., 2017).

AA	Method		S - M	SED <sup>2</sup>	90 % CI		EQ <sup>3</sup>
	Single	Multiple			Lower	Upper	
Essential AA							
Arg	5.35	5.26	0.09	0.15	-0.84	1.03	Yes
His	2.53	2.52	0.01	0.01	-0.03	0.05	Yes
Ile	3.80	4.39	-0.59	0.06	-0.94	-0.24	Yes
Leu	6.11	6.25	-0.14	0.41	-2.70	2.42	No
Lys	8.81	8.55	0.26	0.06	-0.10	0.62	Yes
Met	3.14	3.77	-0.62	0.47	-3.58	2.34	No
Phe	6.49	6.58	-0.08	0.24	-1.61	1.45	No
Thr	5.41	5.34	0.07	0.03	-0.13	0.26	Yes
Trp	4.76	4.95	-0.19	0.27	-1.90	1.52	No
Val	4.65	4.75	-0.10	0.04	-0.38	0.18	Yes

<sup>1</sup>Trial B: Foskolos et al., (unpublished data). n=2 for all comparisons.

<sup>2</sup>Standard error of the difference.

<sup>3</sup>Equivalence determined from 2 one-sided paired t-tests. Methods deemed to be equivalent if 90% CI falls within defined equivalency of -1.5 to 1.5 g/100g of AA.

#### Implications for AA Predictions in Mathematical Nutritional Models

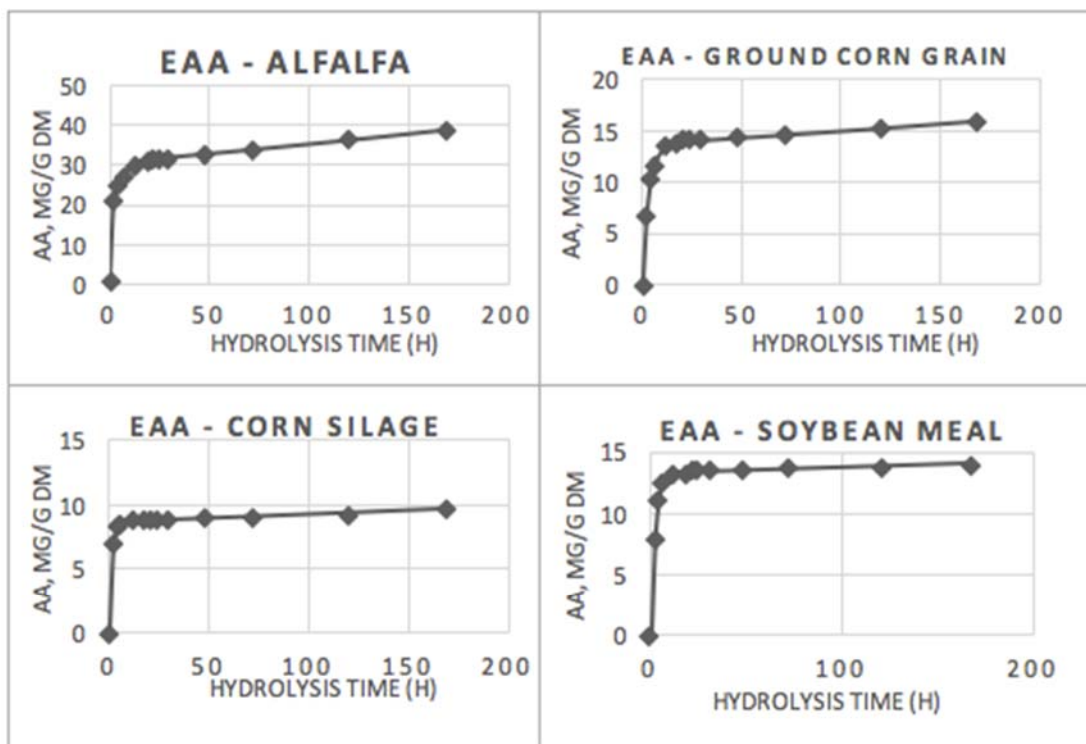
The non-equivalence of the determination methods are important to consider when developing models that rely on AA profiles of microbial protein and feedstuffs. The results from this study and the Rutherford (2009) data indicate that specific AA, especially Ile, Leu, Met, and Val could be underestimated in many post-ruminal AA flow studies when utilizing single time point hydrolysis between 21 and 24 h. This consideration should be recognized when literature values for AA are used in development and evaluation of nutritional models that seek to accurately predict AA supply, especially those that utilize mechanistic post-absorptive sub-models. For example, in this analysis Met was determined to contribute more to total AA than has previously been reported. Currently, the CNCPS v.6.55 uses a profile that corresponds to approximately 1.2% of microbial AA as Met (Higgs et al, 2015; Van Amburgh et al., 2015). Compared with the current analysis (4.7 % of total AA), predictions of AA supply from the model would be expected to increase more than 2 fold (assuming microbial AA accounts for 50% of total AA). Adoption of these values will likely result in a re-evaluation of many common ratios and relationships currently used to balance essential AA for lactating cattle. Given the data presented here and by Rutherford (2009), this might also be true for many of the EAA. The current data, especially regarding the branched-chain AA, would help explain the prediction bias for those AA observed in CNCPS v.7 despite the relatively good prediction of NAN (Higgs, 2014; Higgs et al. submitted). Overall, this analysis illustrates how sensitive nutritional models that rely on microbial AA profiles could be to errors in AA

analysis, especially when a single profile accounts for a large portion of the predicted AA supply. Additionally, future studies should evaluate the use of formalin as a microbial preservative if AA analysis or digestibility is considered as an outcome. Model developers should not include any data from procedures that utilize formalin as a microbial preservative, as it will likely lead to biases and poor model evaluation.

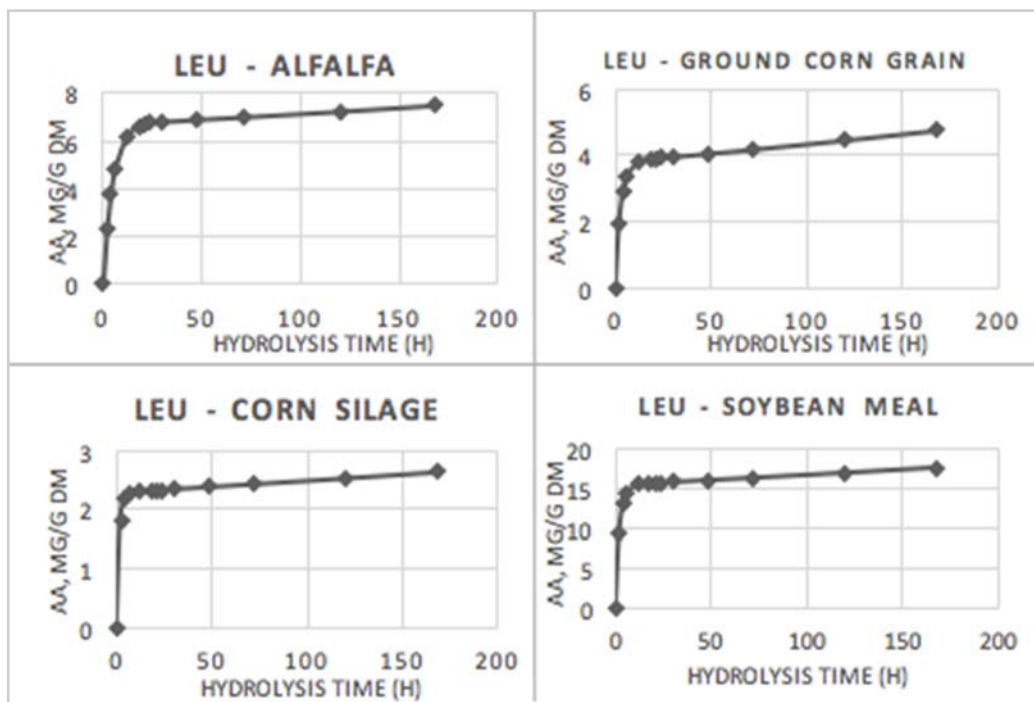
#### Feed AA content

The sum of the concentrations of EAA released from the feeds after multiple hydrolysis times from 24 h to 168 h are presented as least-squares non-linear regression lines, with each data point representing the amount of AA released at each hydrolysis time. The two concentrates and two forages, out of the 26 feeds analyzed, were chosen because they are the most widely used feeds in dairy cattle diets. Overall, the EAA show an increase in release after 24 hr, which resulted in the positive slope observed in the regressions (Figure 1) ( $P < 0.05$ ). The hydrolysis rate for the sum of the EAA ranged from 0.3 to 0.5 mg/h. Of the EAA, the BCAA (Ile, Leu, Val) and Lys are also presented as least-squares non-linear regression lines because of they are the EAA with the greatest increase in release after the 24 hours (Figures 2, 3, and 4). The hydrolysis rate of Leu averaged 0.3 mg/h, except for corn silage that averaged 0.7 mg/h (Figure 2). The hydrolysis rate for Ile averaged 0.3 mg/h for the four feeds in Figure 3. And the hydrolysis rate of Lys averaged 0.45 mg/h, except for corn silage that averaged 1.0 mg/h (Figure 4). For the four feeds represented and the selected AA, it is apparent that there is a continued release of AA from the feed matrix over time and that maximum recoveries of the BCAA and Lys are occurring at very long hydrolysis times with no apparent degradation of the AA.

For the five feeds highlighted in Table 3, the BCAA content increased when measured at the 168 hr hydrolysis endpoint. A similar observation can be seen for Lys for four of the feeds except the corn grain, which remained stable (Table 3). Also, when measured at the longer hydrolysis time point, His content was significantly greater in the blood meal suggesting that blood meal of high digestibility could be a better source of His than currently recognized. The AA content of the analyzed feeds follows the same pattern as that observed in the microbial data, suggesting that the AA content currently being used in the CNCPS feed library needs to be revised again to better reflect the true AA content of feeds. Another update would be a significant undertaking as no database currently exists describing these observations, so many feeds need to be analyzed to fully describe the AA content using the updated approach. Given the uniform response in AA content, either up or down from 21 to 168 hr, it seems reasonable to consider analyzing the AA content at the two time points to characterize feed or other substrate AA content. The least square, non-linear regression takes a considerable amount of time points to use and if the results are uniform, then running simple t-tests or equivalency tests as done in the microbial data might allow us to evaluate more feeds and substrates in a more time and cost efficient manner. Also, if the release of AA from particular substrates is uniform, then it might be possible to apply simple coefficients for specific AA for forages or concentrates for example and this would be very efficient if found to be precise and accurate.



**Figure 1.** Effect of hydrolysis time (h) on release of EAA (mg/g DM) from two concentrates and two forages.



**Figure 2.** Effect of hydrolysis time (h) on release of leucine (mg/g DM) from two concentrates and two forages.



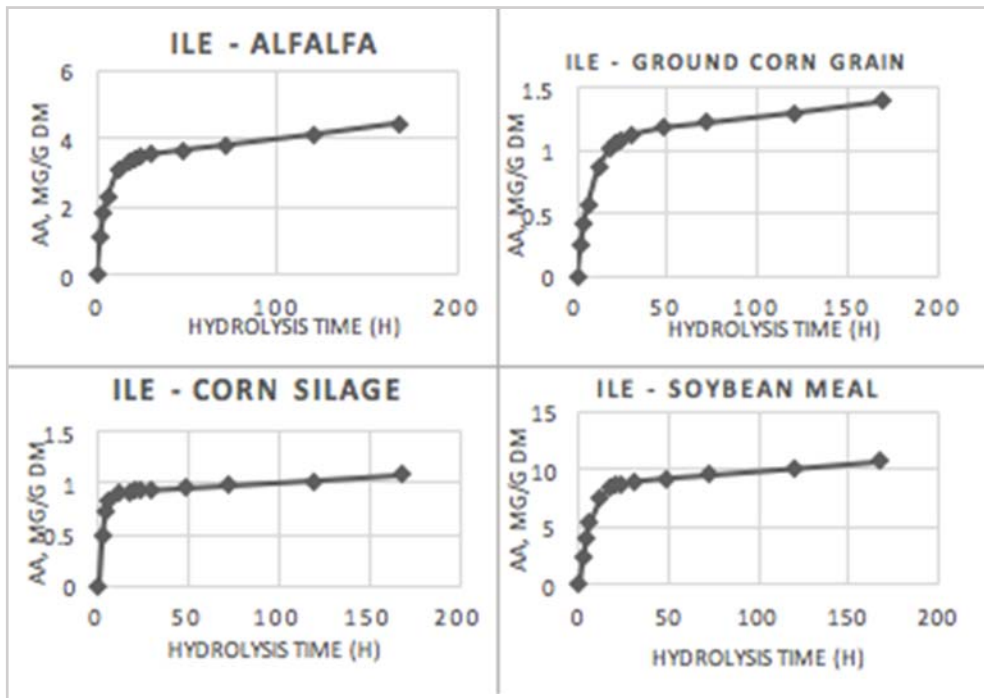
Table 3. The amino acid composition (mg/g DM) of five feeds analyzed at 24 and 168 hr of hydrolysis and content calculated by logistic regression of the content of the residues.

AA <sup>1</sup>	Alfalfa			Canola meal			Ground Corn Grain			Corn Silage			Bloodmeal		
	24h	168h	SEM <sup>2</sup>	24h	168h	SEM <sup>2</sup>	24h	168h	SEM <sup>2</sup>	24h	168h	SEM <sup>2</sup>	24h	168h	SEM <sup>2</sup>
<b>Arg</b>	2.84	3.17	0.17	10.92 <sup>a</sup>	10.59 <sup>b</sup>	0.16	1.77	1.75	0.01	0.54	0.52	0.01	18.13	19.60	0.74
<b>His</b>	1.99	1.94	0.03	6.07 <sup>a</sup>	5.63 <sup>b</sup>	0.22	1.26	1.33	0.03	0.41 <sup>a</sup>	0.33 <sup>b</sup>	0.04	25.20 <sup>a</sup>	30.79 <sup>b</sup>	2.80
<b>Ile</b>	3.45 <sup>a</sup>	4.44 <sup>b</sup>	0.49	5.88 <sup>a</sup>	7.37 <sup>b</sup>	0.75	1.08	1.39	0.15	0.92 <sup>a</sup>	1.07 <sup>b</sup>	0.08	2.75	3.02	0.14
<b>Leu</b>	6.74	7.48	0.37	11.26 <sup>a</sup>	12.69 <sup>b</sup>	0.72	3.91 <sup>a</sup>	4.75 <sup>b</sup>	0.42	2.33 <sup>a</sup>	2.63 <sup>b</sup>	0.15	48.44 <sup>a</sup>	59.99 <sup>b</sup>	5.77
<b>Lys</b>	3.74 <sup>a</sup>	4.56 <sup>b</sup>	0.41	8.66 <sup>a</sup>	9.83 <sup>b</sup>	0.58	1.10	1.40	0.15	0.55 <sup>a</sup>	0.71 <sup>b</sup>	0.08	35.88 <sup>a</sup>	43.55 <sup>b</sup>	3.83
<b>Phe</b>	5.91 <sup>a</sup>	7.25 <sup>b</sup>	0.67	8.76 <sup>a</sup>	9.20 <sup>b</sup>	0.22	2.01	2.36	0.17	1.86 <sup>a</sup>	2.07 <sup>b</sup>	0.10	34.65	35.45	0.40
<b>Thr</b>	3.45 <sup>a</sup>	4.44 <sup>b</sup>	0.49	5.88 <sup>a</sup>	7.37 <sup>b</sup>	0.75	1.08	1.39	0.15	0.92 <sup>a</sup>	1.07 <sup>b</sup>	0.08	2.75	3.02	0.14
<b>Val</b>	4.73 <sup>a</sup>	5.54 <sup>b</sup>	0.41	8.22 <sup>a</sup>	9.39 <sup>b</sup>	0.59	1.66	1.85	0.09	1.35 <sup>a</sup>	1.51 <sup>b</sup>	0.08	29.76 <sup>a</sup>	41.54 <sup>b</sup>	5.89
<b>Met</b>	2.20	2.41	0.11	5.49	5.40	0.04	0.94	0.92	0.01	0.81	0.83	0.01	8.78	8.75	0.02
<b>Trp</b>	2.87	3.59	0.36	8.39	9.22	0.42	1.25	0.89	0.18	0.53	0.53	0.00	25.14 <sup>a</sup>	56.32 <sup>b</sup>	15.6

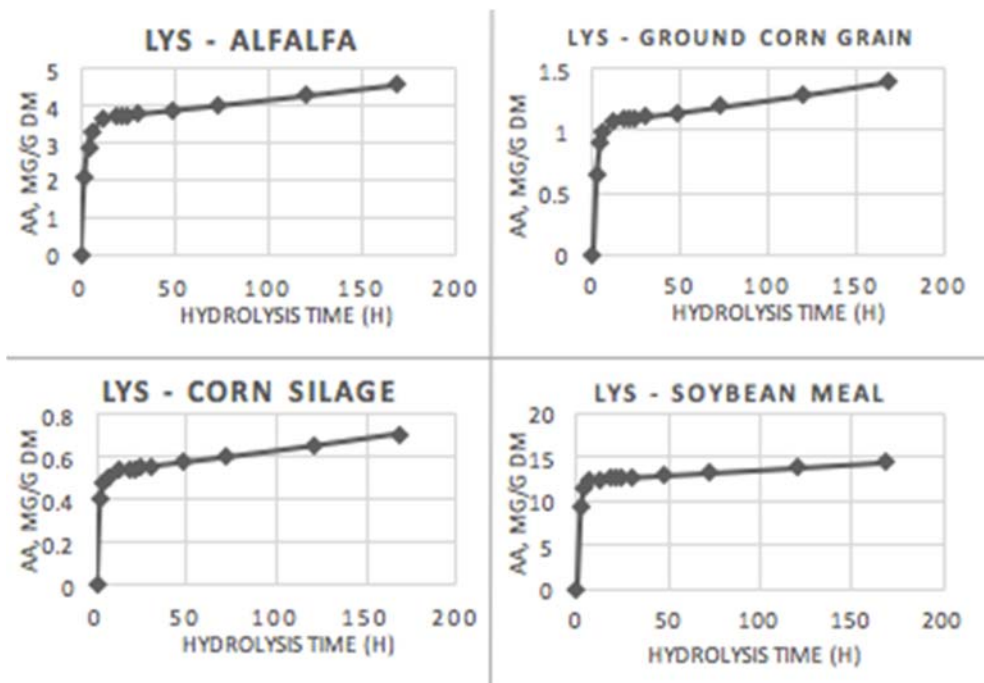
<sup>a, b</sup> Different superscripts for a given feed at 24h vs 168h signifies  $p < 0.05$

<sup>1</sup> AA = Amino acid

<sup>2</sup> SEM = Standard error of the mean



**Figure 3.** Effect of hydrolysis time (h) on release of isoleucine (mg/g DM) from two concentrates and two forages.



**Figure 4.** Effect of hydrolysis time (h) on release of lysine (mg/g DM) from two concentrates and two forages.

## Milk and Tissue AA Content

After analyzing the microbial and feed AA content, it made sense to re-evaluate both milk and tissue, recognizing that the increased AA content of substrates could not be isolated to the supply side of the model. Again, consistent with the previous data, the whole milk from the CURC Dairy demonstrates there is some variability in the AA content of milk when evaluated at the two hydrolysis times. This data has not been fully analyzed and is included for review, as the hydrolysis and integration off the HPLC was finishing as the deadline was approaching. Overall, the data on milk AA content shows some variability and modest increase at 168 hr hydrolysis compared to the 21 hr time point, but differences are smaller than those reported for milk by Rutherford (2008) and much smaller than what was demonstrated for microbes and feeds.

Similarly, the differences in tissue AA content between 21 and 168 hr have not been analyzed, so the data are presented for review and comparison with the supply side information. As with the previous substrates, the tissue BCAA content at 168 hr hydrolysis is generally higher than that observed at 21 hr, whereas the Lys appears to be destroyed at longer hydrolysis times. Thus, for AA like Lys and Met, the 21 hr time point appears to be a reasonable endpoint to ensure optimum and maximum recovery of those AA, especially from tissue, consistent with the original AA methods. This differs greatly from the forages and microbes where Lys release continues without destruction in many of those substrates Table 4. The amino acid composition (mg/g DM) of four whole milk samples taken from the bulk tank at the Cornell University Ruminant Center and analyzed at 21 and 168 hr of hydrolysis and content calculated by logistic regression of the content of the residues. No statistical analysis was conducted on these samples at the time of publication.

Table 4. The amino acid composition (mg/g DM) of four whole milk samples taken from the bulk tank at the Cornell University Ruminant Center and analyzed at 21 and 168 hr of hydrolysis and content calculated by logistic regression of the content of the residues. No statistical analysis was conducted on these samples at the time of publication.

AA <sup>1</sup>	Milk (11.70% MUN)			Milk (8.80% MUN)			Milk (10.10% MUN)			Milk (9.90% MUN)		
	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>
Arg	5.65	6.05	0.43	3.27	4.35	0.36	4.08	4.15	0.48	4.31	5.48	0.31
His	3.22	3.23	0.02	9.87	1.75	3.73	2.77	2.94	0.22	3.46	3.95	0.11
Ile	5.50	5.09	0.09	3.78	5.79	0.59	3.90	6.04	0.62	4.94	7.25	0.45
Leu	11.49	10.97	0.11	8.72	9.24	0.63	9.39	10.69	0.49	10.99	13.59	0.52
Lys	10.02	9.68	0.17	8.65	6.97	0.49	8.02	6.63	0.38	9.66	8.02	0.33
Phe	8.45	8.52	0.07	5.91	6.37	0.18	5.35	7.17	0.81	6.62	7.84	0.25
Thr	5.20	5.08	0.03	3.72	4.60	0.27	3.77	4.37	0.33	4.77	5.66	0.20
Val	7.09	6.64	0.10	4.97	7.12	0.68	5.79	7.44	0.49	6.10	8.63	0.49
Met	5.81	5.78	0.06	4.72	4.72	0.14	4.66	5.05	0.25	6.01	4.68	0.26

<sup>1</sup>AA = Amino Acid

<sup>2</sup>SEM = standard error of the mea

Table 5. The amino acid composition (mg/g DM) of bovine tissues from Diaz et al., (2001) and Meyer (2005) analyzed at 21 and 168 hr of hydrolysis and calculated by logistic regression of the content of the residues. No statistical analysis was conducted on these samples at the time of publication.

AA <sup>1</sup>	Carcass -1			Carcass -2			Head/Hide/Feet/Tail-1			Head/Hide/Feet/Tail-2			Blood/Organs -1			Blood/Organs -2		
	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>	21h	168h	SEM <sup>2</sup>
<b>Arg</b>	11.74	16.20	0.86	15.52	14.96	2.32	20.47	24.55	0.90	17.70	21.89	0.94	8.95	10.19	0.28	8.35	9.74	0.29
<b>His</b>	6.12	7.10	0.22	11.32	8.97	2.64	6.41	6.15	0.09	5.52	5.41	0.17	5.50	6.16	0.15	5.29	6.01	0.14
<b>Ile</b>	4.38	8.06	0.75	11.60	12.11	3.18	4.35	5.91	0.37	3.61	6.10	0.79	3.10	4.31	0.23	3.15	4.53	0.26
<b>Leu</b>	11.75	15.84	0.83	21.42	18.34	4.91	12.35	15.30	0.66	10.82	12.67	0.48	12.96	15.68	0.54	12.23	15.36	0.59
<b>Lys</b>	12.66	11.47	0.33	18.63	12.90	2.86	12.83	10.57	0.46	11.11	9.00	0.46	11.43	9.31	0.42	11.74	9.74	0.51
<b>Phe</b>	9.13	11.22	0.43	11.24	9.90	1.28	11.70	13.37	0.48	9.30	15.80	1.88	9.19	11.49	0.57	8.83	10.54	0.32
<b>Thr</b>	5.74	7.68	0.40	8.32	7.31	1.16	6.48	7.51	0.27	5.63	6.03	0.23	5.85	6.20	0.10	5.56	6.24	0.14
<b>Val</b>	6.89	10.48	0.76	9.93	9.68	1.51	7.94	10.30	0.48	7.02	8.65	0.40	8.48	10.56	0.41	7.76	10.37	0.50
<b>Met</b>	8.24	8.38	0.19	8.78	8.17	0.31	5.17	6.23	0.54	6.29	5.18	0.55	4.41	5.58	0.57	4.46	5.82	0.50

<sup>1</sup>AA = Amino Acid

<sup>2</sup>SEM = standard error of the mean

Overall, this data strongly suggests there are more AA available or contained in the feed, microbes, tissues and milk than the standard AOAC AA method would yield. The implications for model development is significant because the AA content of all feeds in the feed library need to be evaluated against this information. Further, this calls into question the availability of these AA and whether the animal can realize these AA as the feed moves through the post-ruminal gastrointestinal system and this creates more need to ensure intestinal digestibility is well characterized.

The use of 6N HCl as a hydrolyzing agent was developed many years ago and the AOAC standard hydrolysis time of 21 to 24 hr (Gerkhe et al. 1985) is a compromise where the release of all the AA from the protein is maximized while the degradation of the more acid labile AA is minimized (Rutherford, 2009). When comparing this laboratory hydrolysis of protein to that developed evolutionarily by the mammalian gastrointestinal system, it is not surprising that it takes longer to extract the AA from the complex matrix of carbohydrates, proteins, lipids and mineral. The true stomach of most mammals has multiple enzyme systems available to aid in the extraction and breakdown of proteins such as pepsin, trypsin, chymotrypsin, lipase, lysozyme and amylase among many others. Furthermore, different animals have different gastrointestinal tract conditions for digestion and some are more acidic in nature whereas others are more basic to optimize enzyme activity and digestion. For example, the pH optimum for many of the enzymes in the ruminant intestine is more acidic (~pH 5) than the pH required for optimum function in the chicken (pH ~7). Thus, depending upon the species, not only are there enzymes available, but the pH of the system varies in order to improve the efficiency of digestion, something not considered in the laboratory procedures for AA analysis.

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## LAB-BASED MEAT PRODUCTION: SCIENCE FICTION OR REALITY?

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### INTRODUCTION - WHY CULTURED BEEF?

Producing meat through cell culture, currently known as cultured –or clean- meat is being developed as an alternative to livestock produced meat. Starting with the authoritative report by the FAO in 2006, it is increasingly clear that livestock meat production, beef in particular, is unsustainable (FAO, 2006). The 2011 update of that report shows that it is physically impossible to match the 70% rise in demand for meat by 2050 given the constraints of available resources (FAO, 2011). Accepting these constraints and production limits while demand for meat rises, will make meat an increasingly scarce and expensive commodity. Most likely, meat producers will try to serve the demand by increasing production, leading to higher volumes of feed production. Since in factory farming, feed consists mainly of corn and soy, increasing meat production will lead to reduced availability of these staple foods for direct human consumption and thus pose a threat to global food security.

In addition to the threat to food security, it is estimated that between 15 and 20% of all greenhouse gas emission is attributable to livestock farming (FAO, 2006). The globally accepted urgent need to curb climate change further challenges livestock meat. It is also noted that the growing global appetite for meat drives deforestation. Although these environmental effects depend on regional conditions for keeping livestock and on the intensity of livestock farming, their global significance is scientifically accepted.

The third reason for looking at meat alternatives is the growing care of consumers for animal welfare (Dawkins, 2006; De Backer and Hudders, 2015). This is exemplified by the recent ban on caged eggs in the EU following a massive change in consumer behavior favoring free range eggs, despite their marginally higher price. Most, if not all, of the animal welfare issues in the bio-industry are related to the high intensity of farming and the need for cost-effective production. Thus, meat alternatives from partially animal-free production will lead to improved animal welfare, simply by reducing the number of animals in the bioindustry.

While culturing meat might theoretically be a solution for all of these challenges, the technology is still in its infancy and requires appreciable research effort and investment to become a reality. Originally derived from medical technology and still exercised mainly by biomedical investigators, for the technology to be fully developed and integrated, multidisciplinary networks, including cell biologists, biochemists, bioprocess engineers, animal scientists, meat scientists and social scientists, are required.



## TECHNOLOGY

The technology to culture meat is derived from medical tissue engineering and is based on large scale cell culture of satellite cells that can subsequently be differentiated into skeletal muscle (Seale et al., 2000) and fat tissue (fig 1).

The process starts with harvesting satellite cells from a small piece of muscle obtained by a transcutaneous needle biopsy of a cow (fig 1). The stem cells are retrieved by mechanical and enzymatic disruption of the muscle fibers, allowing satellite cells to grow out. This routinely results in a more than 95% purity of satellite cells. The satellite cells start to divide and produce myoblasts. Further multiplication of these myoblasts can be stimulated by standard cell culture techniques. This replication stage is the most resource intense stage, where the cells need to be fed a nutrient-rich fluid a.k.a. “medium”. Medium contains all necessary nutrients for cells to grow. It typically is supplemented with a 10-20% serum, derived from calf blood. Successful efforts have been made to replace the serum by serum-free or chemically defined medium. Myoblasts have sufficient but limited replicative capacity with a maximum of 45 doublings being reported in human cells (Hughes et al., 2015), although this can probably be extended (Magalhaes, 2014). Without extension, this would theoretically allow the production of several hundred kg of meat from one biopsy; more, when the number of doublings is increased. Myoblasts are, like most mammalian cells, “anchor dependent” meaning that they grow when attached to a surface such as bottom of a culture flask or Petri dish.

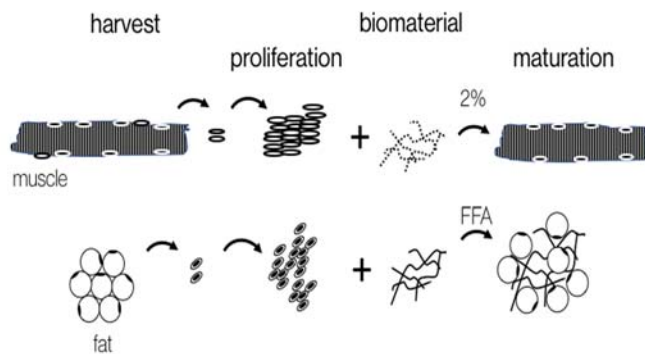


Figure 1. Schematic representation of meat culturing process. It starts with harvesting the satellite cells from muscle and the adipose tissue derived stem cells from fat. The proliferation results in the multiplication factor. Combined with a suitable biomaterial, the tissue matures in either a full-fledged muscle fiber with 2% serum or fat tissue under the influence of free fatty acids (FFA).

Once enough myoblasts are obtained (trillions), the cells are separated in batches of 1.5 million cells that are packed together in a temporary supporting gel, usually a bio-based material such as bovine collagen or fibrin. Reduction of serum in the medium will trigger the myoblasts to merge and form myotubes. By interaction with the gel, the

myotubes start to align and compact to form a tissue in a process that is commonly referred to as (collagen) gel contraction. Currently of animal origin, the gel is gradually replaced by alginate that is functionalized by small peptides such as RGD. Slowly, the myotubes start to contract and if the tissue is constructed in a way that anchors the ends of the myotubes, they develop tension. The tension is the biggest trigger for protein synthesis (Vandenburgh et al., 1999). The easiest scalable format to anchor the muscle fibers is by letting them grow in a ring around a central column of elastic material, thus “self-anchoring” the muscle fiber. Full maturation of the muscle fiber takes about 3 weeks, after which they are ready to be harvested. When ready, the muscle fibers are 2-3 cm long but less than 1 mm in diameter. Ten thousand of these fibers make up a hamburger patty of approximately 100 grams.

Meat also has fat tissue and fibrous tissue, which needs to be created. Fibrous tissue may not be important for a minced meat product, but will be important to recreate full thickness pieces of meat. Fat however is important for taste and texture and arguably, nutritional value. Fat tissue can be generated from a variety of stem cells, including adipose tissue derived stem cells (ADSCs). Alternatively, and more practical, the satellite cell can be used as the source for adipogenesis (Teboul et al., 1995; Asakura et al., 2001). The differentiation of satellite cells into fat cells requires stimulation transcription factor PPAR $\gamma$ . Naturally occurring fatty acids (FFA) are PPAR $\gamma$  and can be used to differentiate stem cells into fat cells. A large number of FFAs were tested and especially pristanic and phytanic acid are in our hands very effective in stimulating adipogenesis. Like muscle cells, fat cells require a temporary support gel to create a tissue, but they do not align and they do not interact appreciably, suggesting that the composition of the gel is less critical than for myoblasts.

Minced meat is currently the focus of research and development, simply because existing technology suffices to create such a product. To reform livestock meat production into a sustainable and environmentally and animal friendly manner it is essential that other types of meat are replaced as well. Creating full-thickness cuts of meat requires a more complex tissue engineering approach. Most importantly, a high-density channel system needs to be incorporated that allows perfusion of medium to every corner of the tissue, so that oxygen and nutrient delivery as well as waste removal are assured. Second, a more complex and larger 3D structure needs to be created with morphological, biochemical and mechanical features tailored for the specific cuts, but also to the needs of the cells. This can be done through 3D printing or any other type of 3D free form fabrication. Third, myoblasts, fat cells and fibroblasts (creating fibrous tissue) need to be co-cultured and differentiated along their respective lineages. These challenges are shared with tissue engineering for medical purposes. Therefore, scientific advances from both areas will lead to an eventual successful strategy, but it will take more time and effort to realize than the minced meat product (Post, 2014).

## OPTIMIZING THE PRODUCT

Culturing meat aims to create the same tissue that consumers appreciate as meat. This is specified not only in its eventual sensory qualities but also in nutrition and health characteristics. Cell and tissue culture will lead to a similar product but with different feed and external mechanical and dynamic environment, it is not immediately obvious that the tissue will be the same. Fortunately, all these conditions are controlled and can therefore be optimized. For a minced meat product, the two components that need to be optimized are the protein and fat composition.

### Protein composition

Muscle tissue is protein-rich. Quantitative analysis of the exact protein composition of muscle turns out to be quite challenging, because it contains up to 6500 proteins spanning several orders of magnitude in expression levels (Ohlendieck, 2011). A large fraction of the weight, contain membrane bound and not soluble proteins, further complicating the extraction of proteins prior to analysis by for instance mass spectrometry. Not only is the range of expression wide, the distribution of protein sizes is also extremely spread out, with muscle tissue containing some of the largest proteins such as titin (12,00 Kd) and nebulin (600-800 kD). The most abundant proteins are myosin, actin and titin, together making up 75% of the cytoskeletal proteins of the muscle cell (Robson, 1995), which by themselves constitute between 40 and 60% of the total amount of protein (Murgia et al., 2015). Whether it is 40 or 60% depends on the type of muscle fiber, i.e. type 1, 2A, 2X or 2B. Cytoskeletal proteins in muscle cells are highly organized in contractile sarcomeres giving the cells their distinguishing cross striation pattern on light and electron microscopy. Highly aligned and tightly co-expressed myosin and actin molecules likely contribute to the texture of meat. Nutritional value, taste and mouthfeel are co-determined by the amount and composition of protein in the muscle cells, so a thorough understanding of the muscle proteome is essential for the development of a product that is aimed to substitute meat. Likely, the most abundant proteins contribute more to taste and texture of meat than the scarcer proteins, although it is possible that some very distinct proteins have specific components contributing disproportionately to taste and appearance. One such class of components is the group of heme containing proteins, with hemoglobin and myoglobin as their prototypic exponents. Hemoglobin and myoglobin are partly responsible for the red color of beef and also for its browning upon oxidation (Pearson and Dutson, 1994; Pearson and Gillett, 2012). Hemoglobin and particularly myoglobin has been associated with a serum-like taste and metallic mouthfeel of beef, so their presence may be important for sensory quality (Miller, 2012).

Although no detailed proteomic analysis has been performed on cultured beef yet, muscle fibers from cultured meat had the typical cross striation indicating sarcomere development (Boonen et al., 2009; Boonen et al., 2011). The muscle fibers also show spontaneous contraction, which is enhanced upon electrical stimulation (Langelaan et al., 2010). The overall protein content is 20% like native muscle fibers (unpublished). The very limited sensory experience of tasters at a public launch of a hamburger of cultured beef in 2013 in London, ascribed a meat taste to the product, with no off flavors, further

suggesting that protein composition is sufficiently replicated. The tasters also described the structure being like ground meat. The muscle fibers however were still yellowish in color because of low myoglobin expression and complete lack of blood and therefore hemoglobin, in the production process. By reducing the oxygen concentration during cell culture however, the myoblasts start to express myoglobin to 5-fold higher levels, in accordance with observations in many other muscle cells of vertebrates (Kanatous and Mammen, 2010; Helbo et al., 2013).

## Fat composition

Fat tissue can be cultured from satellite cells (Lepper and Fan, 2010) by stimulating them with FFA and thereby activating PPAR- $\gamma$ . Making tissue of these fat cells is both mandatory and less challenging than making muscle fibers. It is mandatory as mature fat cells are difficult to maintain in an adherent state when cultured under a fluid layer: they surface due to their lower specific gravity than the watery medium. In a matrix of biomaterial, the cells stay nicely bound and form fat tissue. Unlike the formation of muscle fibers, where the interaction between differentiating myocytes with their matrix is crucial to form the structure, this interaction does not seem to be necessary to produce proper fat tissue, although the level and speed of adipogenesis depends on the matrix characteristics. The fatty acid composition of the cultured fat tissue has not been analyzed yet. For hamburgers, the separate culture of muscle tissue and fat tissue, that is later combined when patties are made, it is very easy to precisely titrate the amount of fat that will be present. Optimizing the production of fat tissue for a hamburger application is still in early development and will require additional work.

## Whole cuts of meat

Processed meat is a sizable part of the entire meat market, but to achieve the goal of producing meat without threatening food security and with minimal environmental burden, it is necessary to create whole cuts of meat, such as a filet mignon or a ribeye steak. The challenges of engineering large tissues are shared with medical tissue engineering and are threefold:

1. Creating a large 3D structure of biomaterials, also known as freeform fabrication.
2. Co-culturing various cells with different culture condition requirements.
3. Formation of a channel and perfusion system for mass transport of oxygen and nutrients and waste removal.

In fact, many technologies for fabrication of 3D structures have already been developed from a variety of human cells. These include but are not limited to 3D printing, lithography, casting and moulding (Houben et al., 2016). Many biocompatible and usually biodegradable materials that are usually synthetic polymers have been investigated and developed for these functions. For food applications, the focus should be on bio-based materials such as alginates, gelatins, cellulose and chitins. In addition to the classic tissue engineering requirements for biomaterials such as mechanical properties, timely degradation and interactivity with cells, the materials or their degradation need to be safe for consumption and produce no off-taste. The freeform fabrication method of choice

needs to be scalable, extremely cheap (compared to medical applications), resource-efficient and sustainable, i.e. seemingly unlimited supply. The condition of unlimited supply can be achieved by using abundant natural materials or by recyclable materials.

Co-culturing and co-differentiating cells of different origin with distinct differentiation protocols, such as muscle cells, fibrocytes (forming fibrous tissue) and fat cells, can be challenging. Our lab has experience with co-culture of fibrocytes and endothelial cells (blood vessels) (Pullens et al., 2009) and several co-culture protocols that include muscle cells have been developed elsewhere (Lesman et al., 2014; Cerino et al., 2016). Obvious co-cultures such as adipocytes and muscle cells have led to novel insights into the reciprocity of these cells, perhaps even leading to new ways to tenderize cultured meat (Choi and Myung, 2014). Thus, this research has challenges as well as opportunities that stretch beyond the cultured meat application

One of the goals in medical tissue engineering of large structures is to create a blood vessels system (Post et al., 2013) that not only facilitates oxygen and nutrient transport to and waste removal from the tissue during culture, but also serves to connect the tissue to the recipient blood supply upon implantation. For the development of whole cut cultured meat, the latter is obviously not required, so it may not be necessary to faithfully recreate a blood vessel system. A much simpler channel and perfusion system might suffice. Much will depend on whether the vascular cells that make up the blood vessels exert an influence on muscle other than just a transport conduit. It is presently unknown if blood vessels contribute to sensory qualities of meat, but it is possible that the vascular cells directly affect muscle cells in a positive or negative manner. We have observed minor effects of cultured endothelial cells on muscle differentiation, when co-cultured in direct contact with each other or with a distance between the cells requiring diffusible substances to drive the interaction (unpublished observations).

Together, the necessary developments to realize whole cut cultured meat are numerous but they will likely be successful and lead to an appropriate gamut of cultured meat products.

## ROAD TO PRODUCT DEVELOPMENT

The development of cultured meat is no longer restricted to academic research. Currently, six companies are busy getting cultured pork, beef and chicken to the market (Brown, 2017). Market introduction will likely still take a couple of years, because the production process is entirely new and not regulated yet. For these products to enter the market, production needs to be scaled and cost-effective and the products must be regulated by governmental agencies.

Large scale cell culture methods have been developed for mammalian cells, but have not been applied yet to their full extent. As most mammalian cells including myoblasts, are anchor dependent, they need to grow on a surface. To allow cell growth in large fermenters that are already available for bacterial and yeast culture, myoblasts will be grown on microcarriers (Moritz et al., 2015). Many microcarriers are commercially available but only few have been tested with myoblasts. Issues such as seeding density,

efficiency of attachment of cells to the microcarriers, bead-to-bead transfer and the efficiency of cell harvest from the beads require extensive research and optimization (Rafiq et al., 2013; Merten, 2015). Not only scalability, but also efficiency and cost-effectiveness of cell culture critically depend on the success of this process as they are intimately related to the maximum achievable cell density. An ideal alternative to microcarrier based myoblast culture would be single cell suspension culture.

The use of fermenters for cell growth and the subsequent use of tissue engineering technology makes producing cultured meat in effect a biotechnology, with more examples in the pharmaceutical industry than in food technology. It is therefore likely that the early companies aiming to bring their products to the market will either retrieve know-how from or form alliances with biotech companies, rather than with traditional food manufacturers.

Safety of novel foods such as cultured meat is of utmost importance to build consumer trust. Governmental regulatory bodies judge if cultured meat is a novel food and therefore requires an assessment of its safety before consumers are exposed. The regulatory process will likely differ from country to country, and will therefore be a factor in determining where market introduction will start. As the final composition of cultured meat is very similar to livestock meat, it is expected that safety tests will be passed.

## CONSUMER ACCEPTANCE AND SOCIETAL CHALLENGES

Cultured meat would need to be widely accepted by consumers in order to be an effective alternative to livestock meat. Surprisingly, a relatively large number of studies has already been focusing on consumer acceptance of cultured –or clean- meat. Different methodologies were used, ranging from focus groups to web-based or cohort-selective surveys. Cohorts from many different countries and continents were surveyed, with somewhat similar outcomes. A sizable minority, in some cases even a majority, of participants expressed willingness to try and/or buy cultured meat when it becomes available. The latest study performed at Maastricht University tested 200 subjects from a cross section of the local population for consumer acceptance, but in the setting of a tasting experience. All 200 subjects sampled a piece of meat that was described as being cultured. There is still notable reservations around cultured or clean meat, but it seems that the obstacles are surmountable given time and thoughtful communication.

## SUMMARY

Culturing meat as an alternative to livestock meat is an exciting technology that could be a solution to the food security and environmental issues associated with the increasing appetite for meat of the global population. The exact protein composition of cultured meat is still unknown although from morphologic observations it can be inferred that the bulk of cytoskeletal proteins will be in the same range as livestock meat. Complementing the cultured meat tissue with cultured fat is in development but still at an early stage. Culturing meat is a challenging technology with still some hurdles ahead before it can be introduced in the market in an appreciable quantity and at a reasonable

price. It is likely that consumers can be enthused for this technology on the basis of its perceived benefits.

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## MILK AVOIDANCE AND MILK ALTERNATIVES

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- The traditional dogma: Lactose maldigesters are lactose intolerant, they need to avoid milk, use digestive aids, take supplements, eat low lactose alternatives and not worry about lower calcium intakes and poor bone health.
- The scientific reality: Perceived lactose (milk) intolerance causes milk avoidance, milk avoidance causes low CA intakes and poor bone health, and lactose maldigestion is easily managed with regular single servings of milk with meals.
- Real or perceived lactose intolerance exists in 5 to 40% of maldigesters, and in a significant number of digesters. These individuals avoid dairy foods resulting in a 200 to 300 mg/day lower intake of calcium. This lower calcium intake reduces bone density and increases the likelihood of fractures.
- Dietary management of lactose maldigestion is relatively easy and depends on: dose, colon adaptation, residual lactase, food sources (with yogurt being very well tolerated), meal feeding and psychological factors (learned aversion).
- Self-described severely lactose intolerant individuals behave just like other maldigesters when blinded to the protocol.
- A serving of milk is unlikely to cause symptoms in maldigesters, particularly when consumed with a meal.
- Calcium intake and therefore bone density depend on perception of lactose intolerance, not reality.
- A significant number of maldigesters and digesters are milk adverse/perceive themselves to be lactose intolerant. This number appears to be increasing.
- Milk adverse individuals do not easily alter their diets to include milk.
- Alternative beverage consumption has increased while milk consumption has fallen dramatically since 1950. This has been most apparent for whole milk. But lower fat milk has not fully replaced the reduction in intake of whole milk.
- While milk consumption has fallen, total dairy intake has remained strong, actually growing due to the large increase in cheese consumption.
- Alternative beverages include carbonated beverages, low lactose milks and plant-based beverages.
- Americans still consume more milk than any other country.
- Carbonated beverage intake grew rapidly from the 1950s to the 1990s, but has fallen substantially in the last 15-20 years.
- The anti-sugar movement appears to have made substantial progress through marketing, taxation (Berkeley, Oakland, Philadelphia, Chicago, San Francisco) despite a large lobbying investment by the carbonated beverage industry.
- Brands of low lactose and lactose-free milks have grown substantially, but still remain a very small segment of the overall dairy market.

- Plant-based beverages sales are growing at double digit rates. Almond beverages have surpassed soy beverages in sales. There are many new product formulations coming on the market. But, overall the segment is still small (less than 10%).
- Almost every nut and grain is being tried in this market. Almond beverages are most popular with an estimated 6.7% of the market followed by soy beverages with 2%, as compared to fluid milks with 81.2%.
- Nutrient composition of plant-based beverages can vary dramatically. Products still market 'milk equivalence' with a plus of being plant-based.
- Consumers are attracted to the plant-based diet focus, anti-dairy sentiment, lactose intolerance and sustainable agriculture arguments.
- Anti-dairy market seems to be one of the primary tools to market plant-based beverages, yet at the same time the products are marketed as equivalent to or better than milk. This is an interesting dichotomy.
- Cherry picking research data is part of the marketing approach.
- Drivers of choice for dairy remain taste, natural, healthy, organic, reduced fat, vitamin fortified and high quality protein. In contrast, drivers of choice for plant-based beverages are taste, healthy, weight control, growth hormone-free, digestion, natural, organic, vitamin fortified, and calcium and protein equal to milk (McCarthy 2017).
- Closing thoughts include:
  - It is not reasonable to expect fluid milk consumption in the US to increase.
  - Global markets have much upside potential.
  - Plant-based diets will likely become more common in developed countries.
  - Is the current US animal production industry sustainable?

# INFRARED MILK FATTY ACID ANALYSIS: EXPERIENCE IN THE FIELD FOR FARM MANAGEMENT

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## INTRODUCTION

In 2014 (Barbano et al., 2014), we introduced the application of mid-infrared (**MIR**) for rapid milk fatty acid (**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/fatty acid). 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 (Woopert 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 (Woopert 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. 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 66.1lb (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.

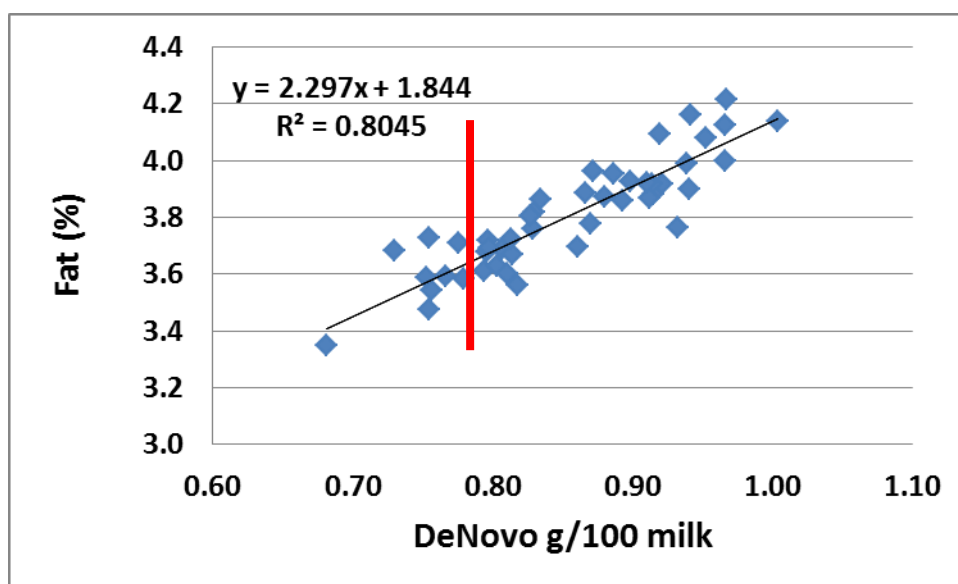


Figure 1. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of de novo FA in milk. In general, a farm needs to have a concentration of de novo FA higher than 0.85 g/100 g milk to achieve a bulk tank fat test higher than 3.75%.

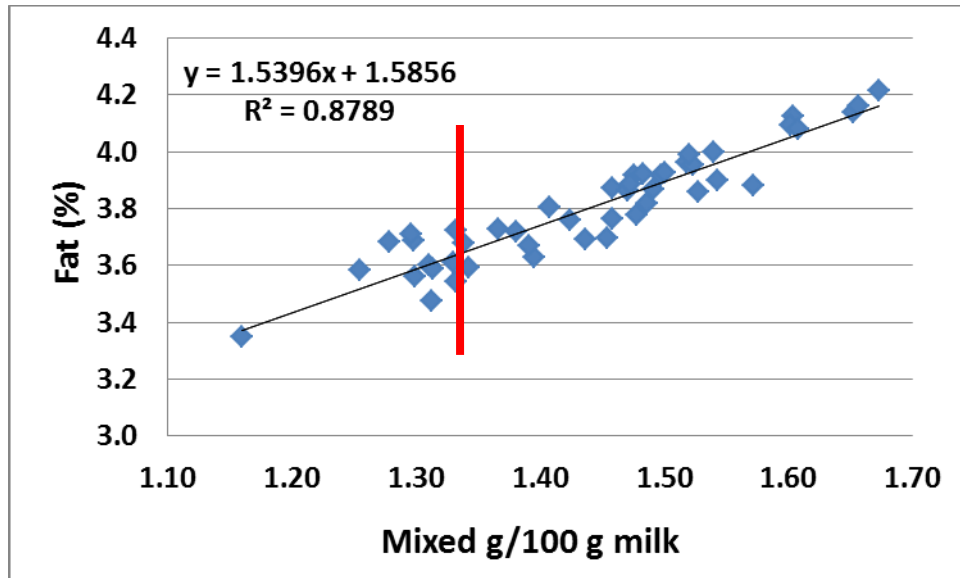


Figure 2. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of mixed origin FA in milk. In general, a farm needs to have a concentration of mixed origin FA higher than 1.40 g/100 g milk to achieve a bulk tank fat test higher than 3.75%.

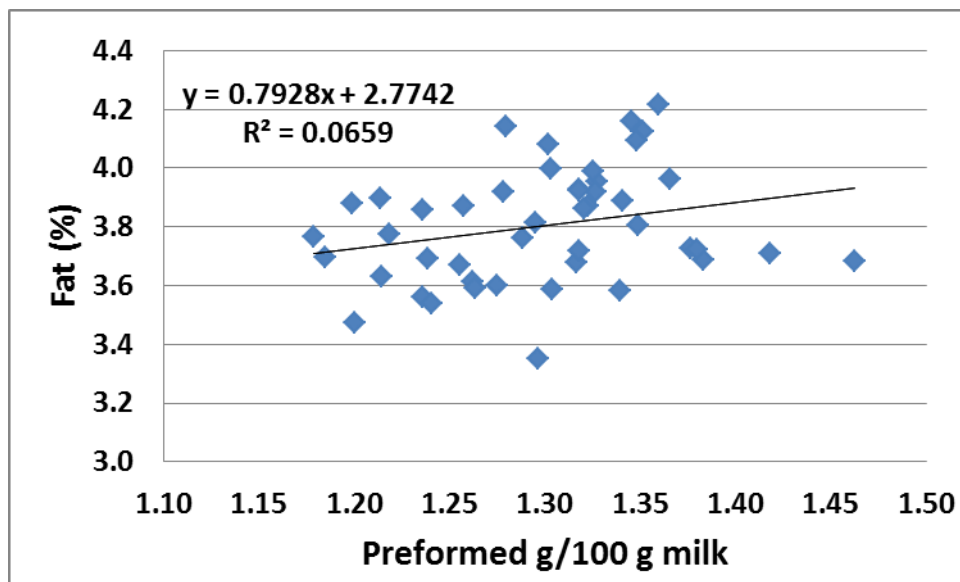


Figure 3. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of preformed FA in milk. In general, the variation in preformed FA concentration in Holstein herds is less than de novo and mixed origin FA and is not well correlated with bulk tank milk fat test.

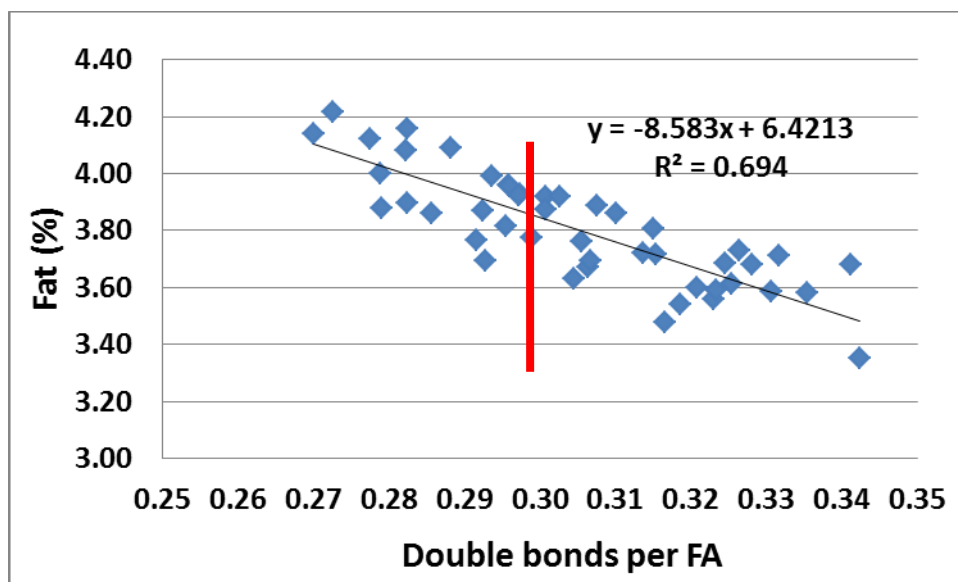


Figure 4. Relationship of bulk tank milk fat FA unsaturation with bulk tank milk fat test. As double bonds per FA increases the bulk tank milk fat test decreases. To achieve a 3.75 % fat test a farm needs to have a double bond per FA of less than 0.31.

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 fatty acid 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. 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.

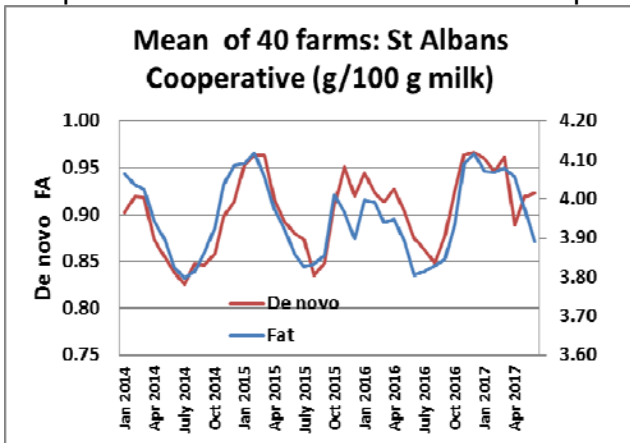


Figure 5. Seasonality of milk fat and de novo FA in milk.

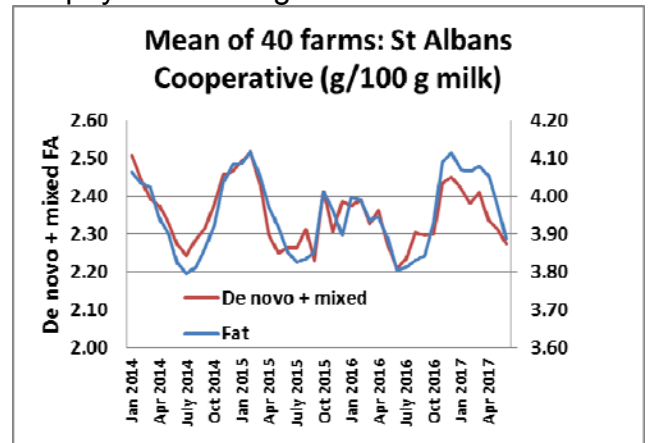


Figure 6. Seasonality of milk fat and de novo + mixed origin FA in milk

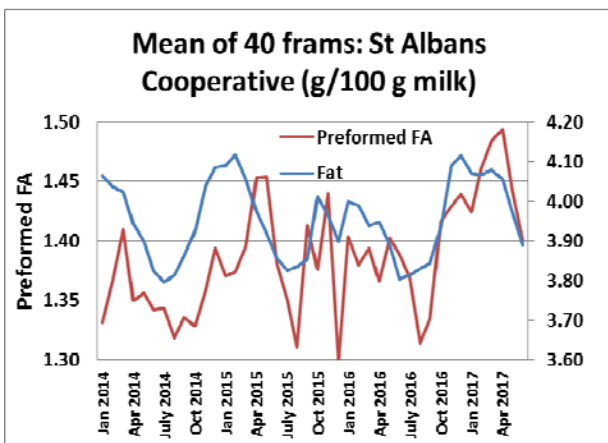


Figure 7. Seasonality of milk fat and preformed FA in milk.

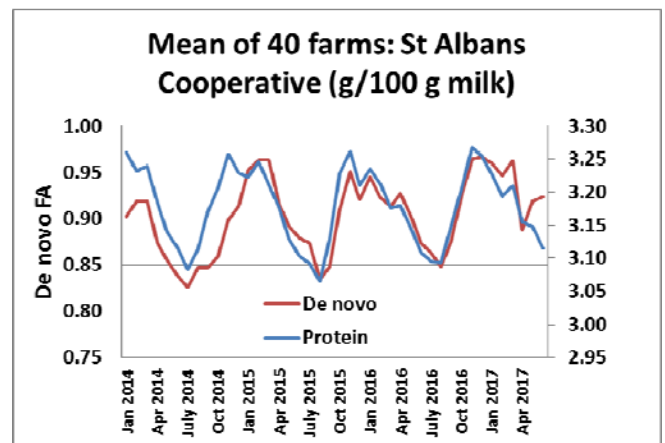


Figure 8. Seasonality of milk protein and de novo FA in milk.



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 (Figures 9-13) below for 167 farms.

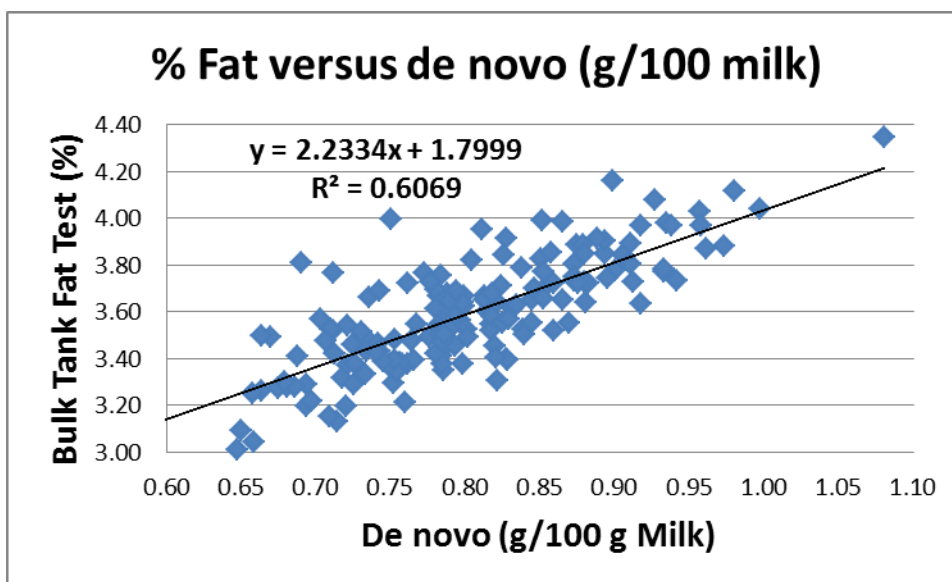


Figure 9. Correlation between bulk tank fat and de novo FA concentration (167 farms).

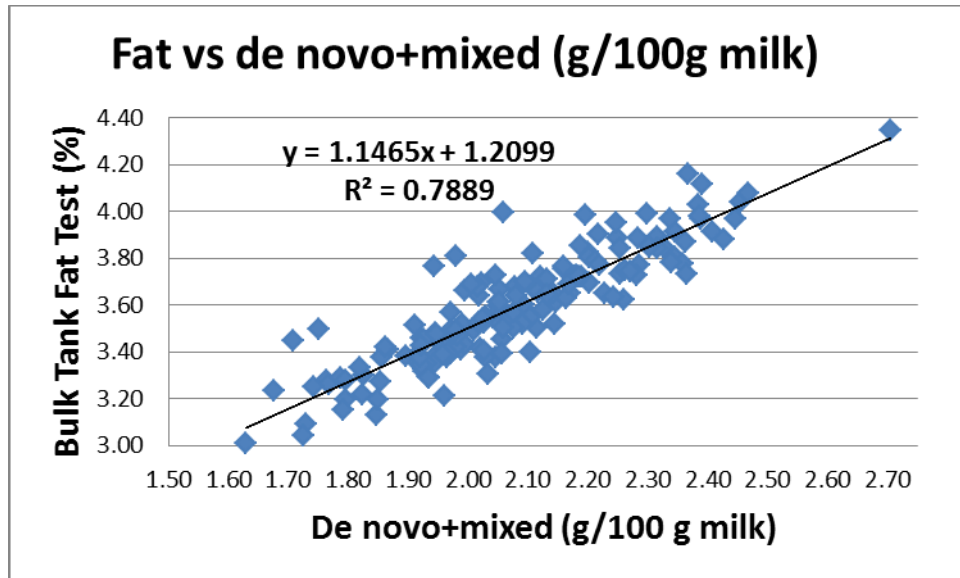


Figure 10. Correlation between bulk tank fat and de novo + mixed origin FA (167 farms).

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 correlated with total weight of milk produced per day. Those relationships are shown below.

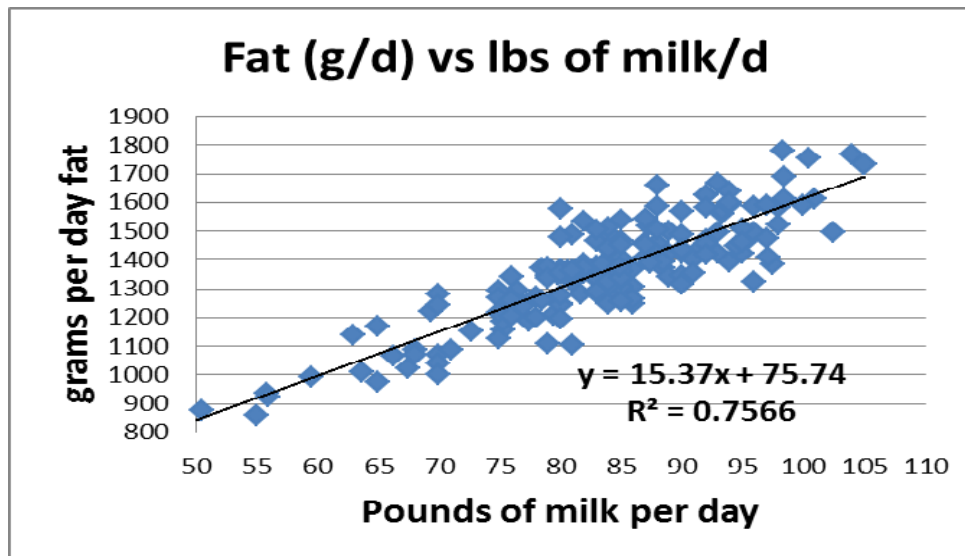


Figure 11. Grams of fat per cow per day and milk production (167 farms).

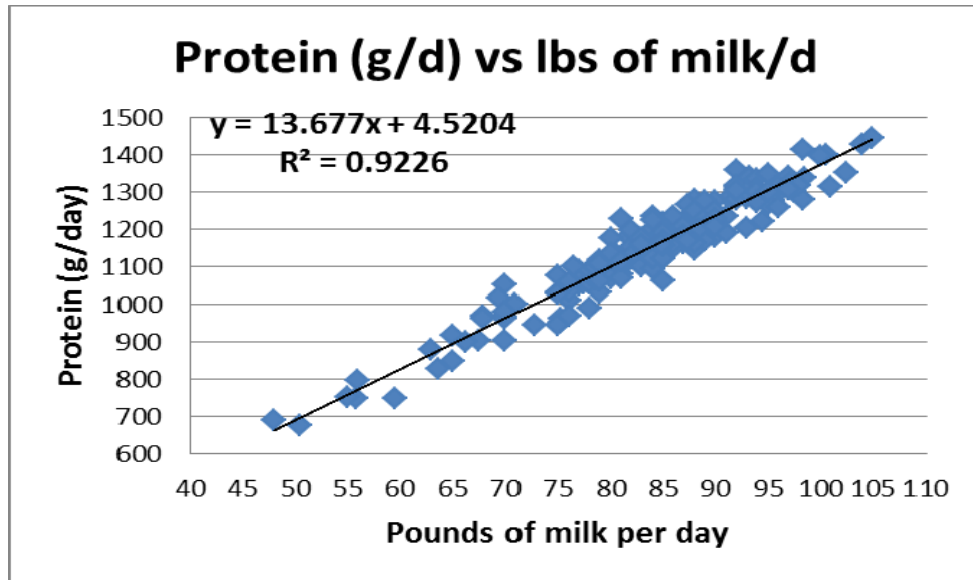


Figure 12. Grams of protein per cow per day and milk production (167 farms).

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 is highly dependent on glucose metabolism in the cow. If you want to produce more milk per cow, you need to produce more lactose per day, as shown in Figure 13 below. 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. How do we feed for that?

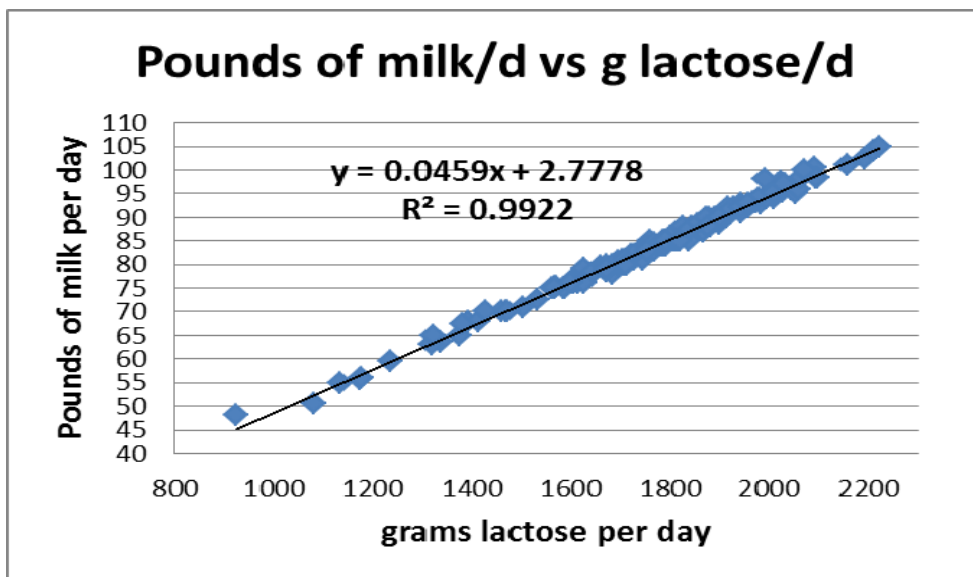


Figure 13. Grams of lactose produced per cow per day and milk production (167 farms).

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 herd producing an average of about 92 lb (41.77 kg) per cow per day on TMR feeding system. The fat, protein, and lactose content of the milk are shown in Figure 16.

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.

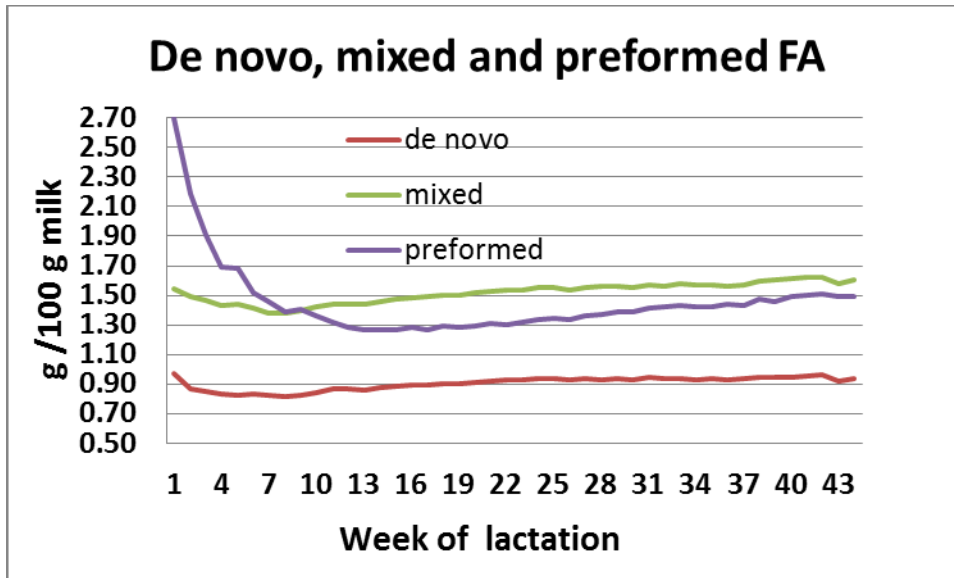


Figure 14. De novo, mixed, and preformed FA (g/100 g milk) over lactation for all cows.

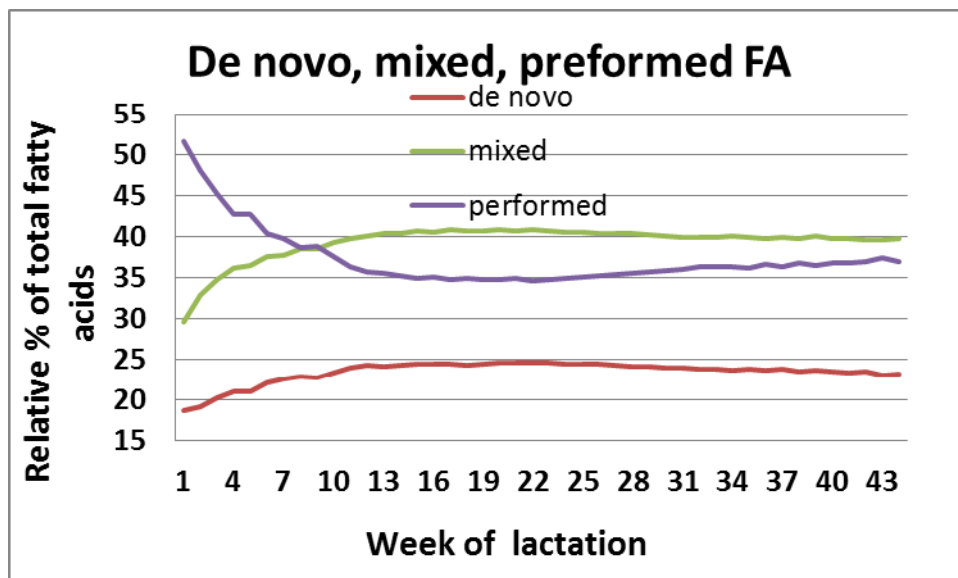


Figure 15. De novo, mixed, and preformed FA (relative %) over lactation for all cows.

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 fatty acids. 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.

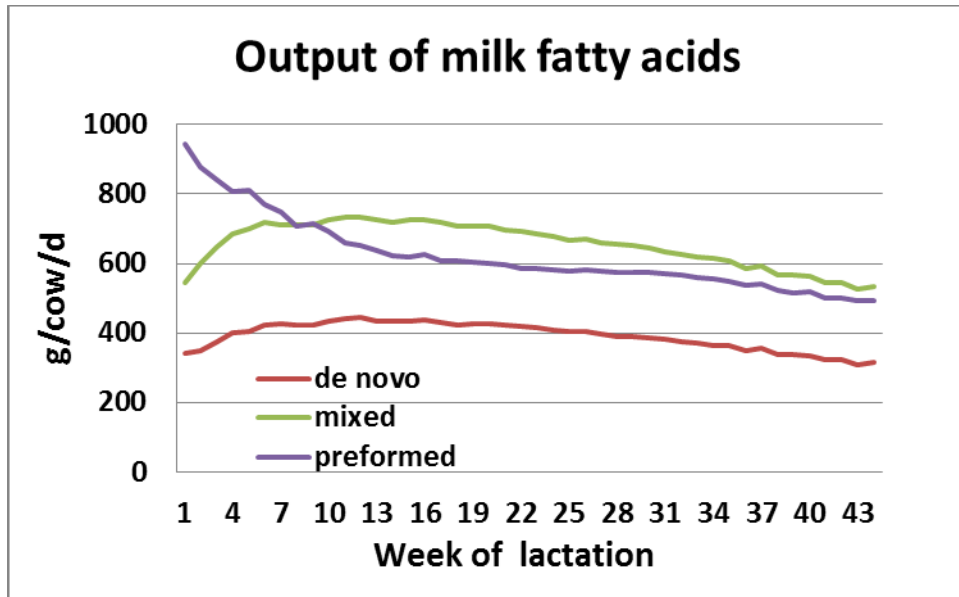


Figure 16. Stage of lactation production graph for all cows (g/cow/day).

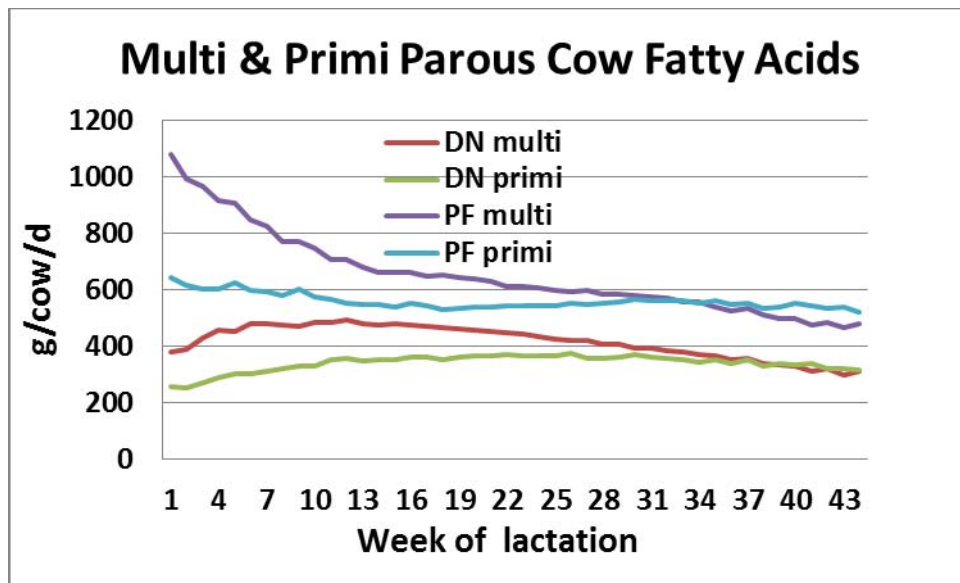


Figure 17. Stage of lactation: de novo and preformed FA for primi and multiparous cows.

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 data 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 fatty acid)
- 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 fatty acids (g/100 g milk and g/day production)
- Milk mixed origin fatty acids (g/100 g milk and g/day production)
- Milk preformed fatty acids (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 mammary 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 Holstein herds is that when the double bonds per FA is > 0.31, the probability of trans FA induced milk fat depression is greatly increased for Holstein milk. 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 need 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 the 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 milk production per cow per day 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 fatty acids: 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 fatty acids 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 their per day output of fat and protein given an adequate supply of their precursors.

*Preformed fatty acids: 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 fatty acid 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 fatty acid 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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# MANAGEMENT AND NUTRITION FOR MILKING SHEEP IN SHORT AND FREQUENT LACTATIONS

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## INTRODUCTION

Traditional sheep dairy production is seasonal. The lack of year-round availability of fresh product has deterred steady sales and buyers' markets of sheep milk and sheep milk products in the US. Research from Europe suggests seasonal income, as well as difficulties to coordinate lambing seasons (Sitzia et al., 2015), as interference to an even and reliable marketplace. The 30% rise in consumption of dairy sheep products in the past 20 years (Thomas, 2014) – the US imports 50 to 60% of the world's annual exports in sheep dairy products (Thomas, 2014) – indicates a dramatic increase in US demand for sheep milk products. Official FAO data from 2011 lists an import amount of 26 million kg of sheep milk cheese. With a cheese yield of ~18-25% that is equivalent to ~123 million kg of raw sheep milk.

A survey conducted prior to this research showed that 70% of the ~60 responding sheep dairy farmers with flock sizes ranging between 20 and 2,000 head in northeastern US indicated that they would consider a change in a current operation and begin to milk sheep year-round to meet this rising demand. Little new East-Friesian or Lacaune dairy sheep breed genetic material is available. Import restrictions from Europe are stringent. Additionally, the non-surgical artificial insemination traditionally used in sheep (Purdy et al., 2009) and a lack of a phenotypic data for a comprehensive genetic analyses limits the pace of selection to meet the rising demand. Hence, research into new management practices, aseasonal breeding ability, and nutritional strategies to boost year-round milk production in sheep is needed to provide dairy sheep farmers with techniques to produce at a price competitive with imports and to allow for consistent income and economically viable operations.

The main aims of an on-going two-year experiment (projected to end September 2018), at the Cornell Teaching barn are: 1) a comparison of published values for 180-day, yearly lactations of traditionally-milked dairy-breed ewes with yearly yields and components of Dorset and Finnsheep × Dorset ewes milked in short and frequent lactations; and 2) the determination of optimal dietary levels of fermentable fiber for maximum milk production, ewe body condition, fertility, fecundity, and flock health.

## EXPERIMENTAL DESIGN

Dorset, Finnsheep and Dorset X Finnsheep crossbred ewes, not previously selected for dairy production, are being milked in short and frequent lactations on the STAR accelerated lambing system, developed by Brian Magee and Doug Hogue at

Cornell in the 1980s (Lewis et al., 1996; Lewis et al., 1998; Posbergh et al., 2017). In the STAR system, any ewe with a perfect conception rate will lamb 5 times in 3 years or 1.67 times per year which results in short and frequent lactations when adapted to dairy production.

The experiment is a triply replicated 3 x 3 Latin square with 3 diets (30, 35, 40% pfNDF) and 3 lactations (1, 2, 3) within each of 3 STAR groups (STARR, STARB, STARG) of ewes. Each Latin Square includes 3 pens with a minimum of 4 ewes within STAR group as columns, lactations as rows and diets repeated orthogonally in each pen and lactation of each Latin Square. Ewes and squares will be the replicates for yield and components of milk with 44 df for error. Squares will be the replicates for feed intake and milk/feed with 18 df for error. The 2 df for diets will be split into linear and quadratic orthogonal contrasts. The error for all response variables will be small because the replicated Latin Square design efficiently removes the group, lactation, and pen sources of variation.

Each STAR group is lambing and lactating consecutively at 205 days intervals. Ewes will lactate in three 73- to 103-day lactation periods throughout this two-year study with rebreeding starting on day 73 of each lactation. Lambs are removed within 12 hours after birth and after receiving colostrum from their dams. They are reared artificially on the cold-milk, lambar system. Breeding is enhanced with teaser rams. Except for the autumn breeding season, CIDRs are used (Inskeep, 2011) to ensure that the maximum number of ewes are cycling at the time of breeding. Milk yields are recorded twice daily for each ewe. Feed and refusal weights are collected twice daily for each dietary group; samples are collected once weekly. Rumen fluid and fecal samples are collected biweekly. Fecal, feed, and refusal samples will be analyzed with the acid insoluble ash method for digestibility (Thonney, 1979). Rumen fluid pH is measured immediately after collection. Then, rumen fluid is acidified and frozen for later VFA determination. The milking ewes are weighed once weekly during lactation. AM and PM milk samples are collected once weekly and analyzed for total fat and protein, SCC, MUN and fatty acid composition with a novel infrared method developed for cow milk and adapted for sheep milk by Dr. Dave Barbano from the Cornell Food Science Department (Woolpert, 2016, 2017).

The data reported here are from the first lactations from each of the STAR groups, STARR, STARB, and STARG, as well as a comparison of 1<sup>st</sup> and 2<sup>nd</sup> STARR lactations. Milk yield for the first lactation curves was fitted in MINITAB 18 and R to a model that include the fixed effects of STAR group, diet, and the STAR group x diet interaction. Ewe was included a random effect nested within STAR group and diet. Linear, quadratic, and cubic polynomials for days in milk (DIM) were included as covariates, allowing for the possibility of different covariates for STAR groups and diets. Non-significant covariates were removed by a modified step-down procedure until the model included only effects with *P*-values < 0.05. The statistical analysis of milk yield for lactations 1 and 2 of STARR was similar.

Dry matter intake for pens was analyzed by analysis of covariance with the effect of STAR group, diet, and the STAR group x diet interaction in the model and linear and quadratic effects day of lactation as covariates. The effects of STAR group and diet on covariates were included when *P*-values were < 0.05.

## Diets

Previous research at Cornell showed that feed intake is impacted by the source and concentration of dietary fiber (NDF) (Schotthofer et al., 2007; Thonney and Hogue, 2007). The effect source of NDF was mainly due to the proportion of potentially-fermentable NDF (pfNDF) compared with indigestible NDF (INDF) (Thonney and Hogue, 2013); where pfNDF is defined as NDF – 1X maintenance INDF, with 1X maintenance INDF being determined by the concentration of indigestible dry matter at 1X maintenance (Thonney, 2017), minus 10 to 15 percentage units of DM as metabolic fecal losses (Van Soest, 1994). The diets were balanced on their carbohydrate fractions, mainly the concentration of pfNDF, followed by crude protein, minerals, and vitamins (Thonney, 2017). Due to their high concentration of pfNDF and pectins (that are fermented similarly to NDF), soy hulls were substituted for corn to increase the dietary pfNDF concentrations (Table ).

Table 1. Composition of initial experimental diets (% of DM). The three diets formulated for this experiment differ in their levels of pfNDF: 30, 35, or 40%, respectively and are fed ad libitum with about 500 g of hay per ewe per day.

Ingredient	30% pfNDF	35% pfNDF	40% pfNDF
Soy hulls	43.60	52.10	60.60
Corn	41.20	33.40	25.60
Soybean meal	10.30	9.91	9.42
Vegetable oil	2.22	2.23	2.23
Cornell sheep premix	1.06	1.06	1.06
Ammonium chloride	0.78	0.78	0.78
Calcium carbonate	0.56	0.33	0.11
Salt	0.22	0.22	0.22
<i>Estimated components</i>			
DM	89.93	89.85	89.77
DDM	79.97	79.95	79.22
CP	16.10	16.11	16.10
NDF	36.37	41.72	47.07
pfNDF	30.61	35.56	40.52
INDF	5.87	6.27	6.66
NSCHO	38.93	33.67	28.44
EE	4.95	4.80	4.65
Ash	4.15	4.19	4.23

## RESULTS AND DISCUSSION

Our findings so far are concurrent with previous research that showed minimal levels of pfNDF are needed to maintain healthy rumen function (Schotthofer, 2007; Schotthofer et al., 2007; Hein et al., 2010). Zero occurrence of acidosis was recorded; measured pH levels for STARR-2 ranged from 6.13 to 7.05. Sheep don't seem to rely on physically effective fiber (Mertens, 1997) and small particle size in feed doesn't appear to prompt acidosis (Nudda et al., 2004). For STARR-1, STARB-1, and STARG-1, pfNDF content of the experimental diets was highly influential ( $P < 0.000$ ) on intake, as well as on milk production ( $P < 0.000$ ). NDF concentrations of 37% of the dry matter were suggested in diets for lactating dairy ewes by Pulina (2004), which is in accordance to our findings but only if the NDF is highly fermentable.

### Feed intake

Dry matter intake in lactation 1 tended to increase slightly with day of lactation but the change depended upon STAR group and diet (Figure 1). The STAR group x diet interaction ( $P = 0.004$ ) intakes adjusted to the mean of 41 days of lactation are shown in (Table). The ewes in STARG were yearlings lambing and lactating for the first time so their lighter weights resulted in lower dry matter intakes. Dry matter intake was lowest for ewes fed the 40% pfNDF diet, but the highest intake varied with STAR group between the 30 and 35% pfNDF diets.

Table 2. Effect of STAR group and diet on daily dry matter intake (kg/ewe) in lactation 1 (SEM = 0.06).

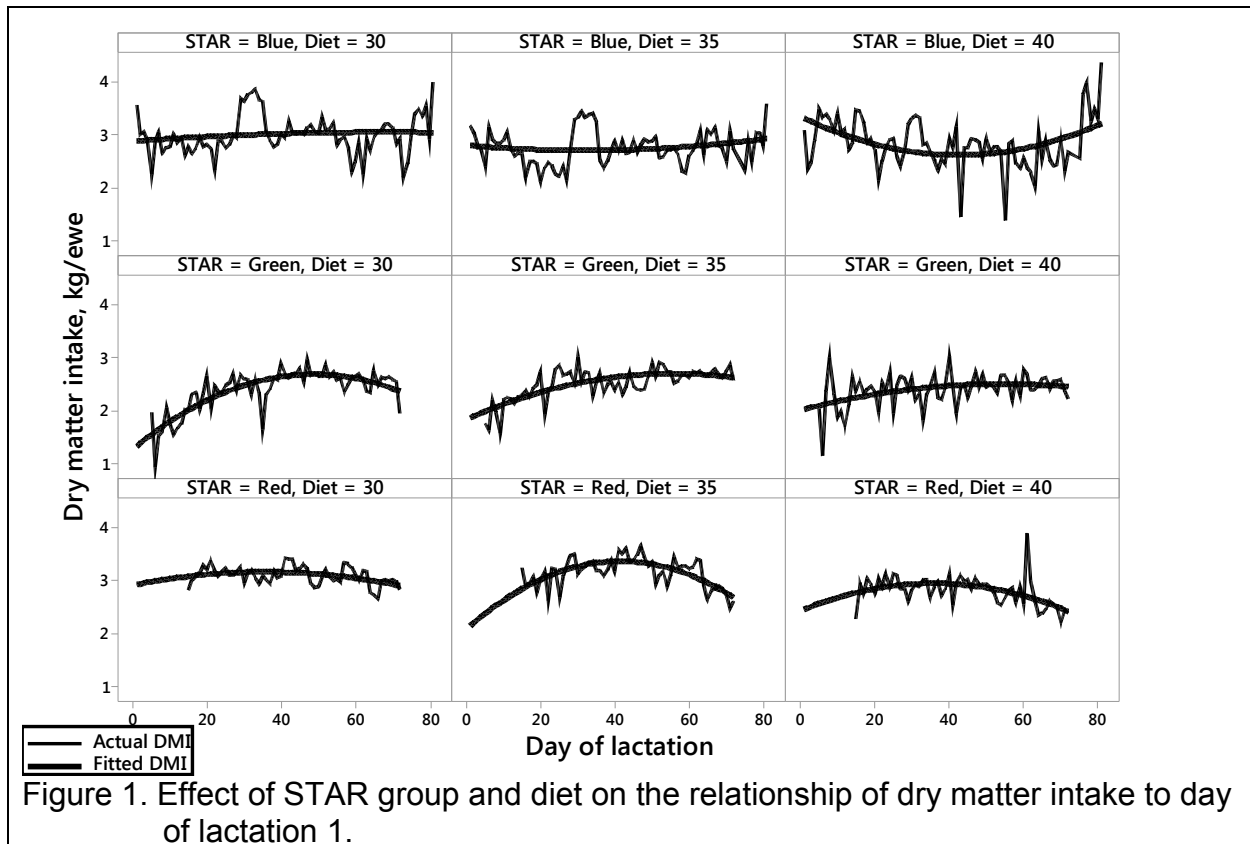
Group and Lactation	Diet		
	30% pfNDF	35% pfNDF	40% pfNDF
STARR-1	3.2	3.4	2.9
STARB-1	3.0	2.7	2.6
STARG-1	2.6	2.6	2.5

### Milk yield

Previous studies measured milk yields of traditional meat sheep a few times during lactation, mainly using weigh-suckle-weigh methods with ewes nursing lambs or in mixed milking and nursing systems. Suffolk and Targhee ewes reached peaks up to 3,744 g/d (Ramsey et al., 1998), and crossbred ewes peaked at 3,680 g/d (Cardellino and Benson, 2002).

The milk production results from the first lactation are summarized in Table 3. The 3<sup>rd</sup> degree polynomial was significant with both STAR group and diet affecting ( $P < 0.001$ ) the equations that described the lactation curves. The first differentials of the average lactation curves for each diet within each STAR group were used to find peak lactation day and yield (Table ). The integrals of the equations were used to calculate 73-day yield (Table ). Previous research with nursing ewes (Schotthofer et al., 2007; Thonney and Hogue, 2007), indicated that ewes fed diets with levels of pfNDF higher

than 30% would produce more milk. In line with the feed intake data (Table), however, peak milk yield day was later and higher and 73-day yield was highest for ewes fed the diet containing 30% pfNDF (Table 3). These results and the relatively high ruminal pH values for all three diets from a one-time sample during the second lactation of the STARR, suggests that 30% pfNDF is sufficient to maintain excellent ruminal function. Therefore, the more highly digestible 30% pfNDF diet allowed for higher milk production.



The highest yielding Cornell ewes in our management system, so far, produced 2,722 g/d (STARR-1), 4,544 g/d (STARB-1), 2,903 g/d (STARG-1), and 4,082 g/d (STARR-2). In longer dairy sheep lactations, 25% of the milk yield achieved throughout lactation occurs within the first 30 DIM (Folman et al., 1966). Milking sheep in short and frequent lactations provides the opportunity to skim the lactation curves around their peaks, ranging between 7 and 30 days (Cardellino, 2002; Peterson, 2005) and makes use of high peak yields from the first part of lactation. Total milk yields of dairy sheep are compared with Cornell Finnsheep x Dorset crossbred ewes in Table . Overall through this stage of the experiment, the 30% pfNDF diet resulted in the highest response in milk production. All ewes fed this diet are listed in Table as Finnsheep x Dorset diet 30 with high producing ewes potentially selected for breeding listed as Finnsheep x Dorset High 30. Lactation lengths for the Finnsheep x Dorset diet 30 ewes are a multiple of 1.67, the maximum possible per year on the STAR system.

Table 3. Effect of STAR group and diet on milk production in lactation 1.

Group		Diets		
		30% pfNDF	35% pfNDF	40% pfNDF
STARR-1	Peak day	26	17	0
	Peak yield, kg/ewe	1.27	1.03	0.95
	73-d yield, kg/ewe	86	69	46
STARB-1	Peak day	12	14	1
	Peak yield, kg/ewe	2.59	2.11	2.05
	73-d yield, kg/ewe	144	116	103
STARG-1	Peak day	28	25	2
	Peak yield, kg/ewe	2.09	1.62	1.28
	73-d yield, kg/ewe	138	102	63

Although diets had a substantial influence ( $P < 0.001$ ) on lactation curves throughout all STAR groups and lactation periods (Table ), there was considerable variation among ewes within all groups and between STARR-1, and STARR-2. Example lactation curves for STARR-1 (24 October 2016 – 11 January 2017), and STARR-2 (3 June 2017 – 17 August 2017) groups are shown in Figure 2. The prominent differences between the milk yields of lactation 1 and 2 within all diets likely stem from the different management the ewes underwent prior to entering this study.

Table 4. Milk yield comparison among dairy and meat breeds.

Sheep breeds	Lactation length d/year	Milk yield kg/year	Literature
East Friesian	189	359	(Thomas, 2014)
Lacaune	180	345	(Thomas, 2014)
Finnsheep x Dorset Diet 30	125	225	Current experiment
Finnsheep x Dorset High 30	115	246	Current experiment

#### Ewe weight

The ewes in all groups gained weight after parturition and maintained excellent body condition and health while achieving high levels of milk production, even in the first part of lactation. For STARR-1, weight gain was significantly high ( $P < 0.001$ ) throughout lactation. As can be observed in Figure 2, STARR ewes produced dramatically less milk in their first lactation compared with their second lactation. This might be related to the diet (pasture, no concentrate) during breeding and gestation prior to enrollment in this study and their first lactation. Body weight and condition during breeding are very influential on milk production in the successive lactation period (Reynolds, 1991). Combined with our results, this suggests that ewes during breeding need well-balanced, highly digestible diets.



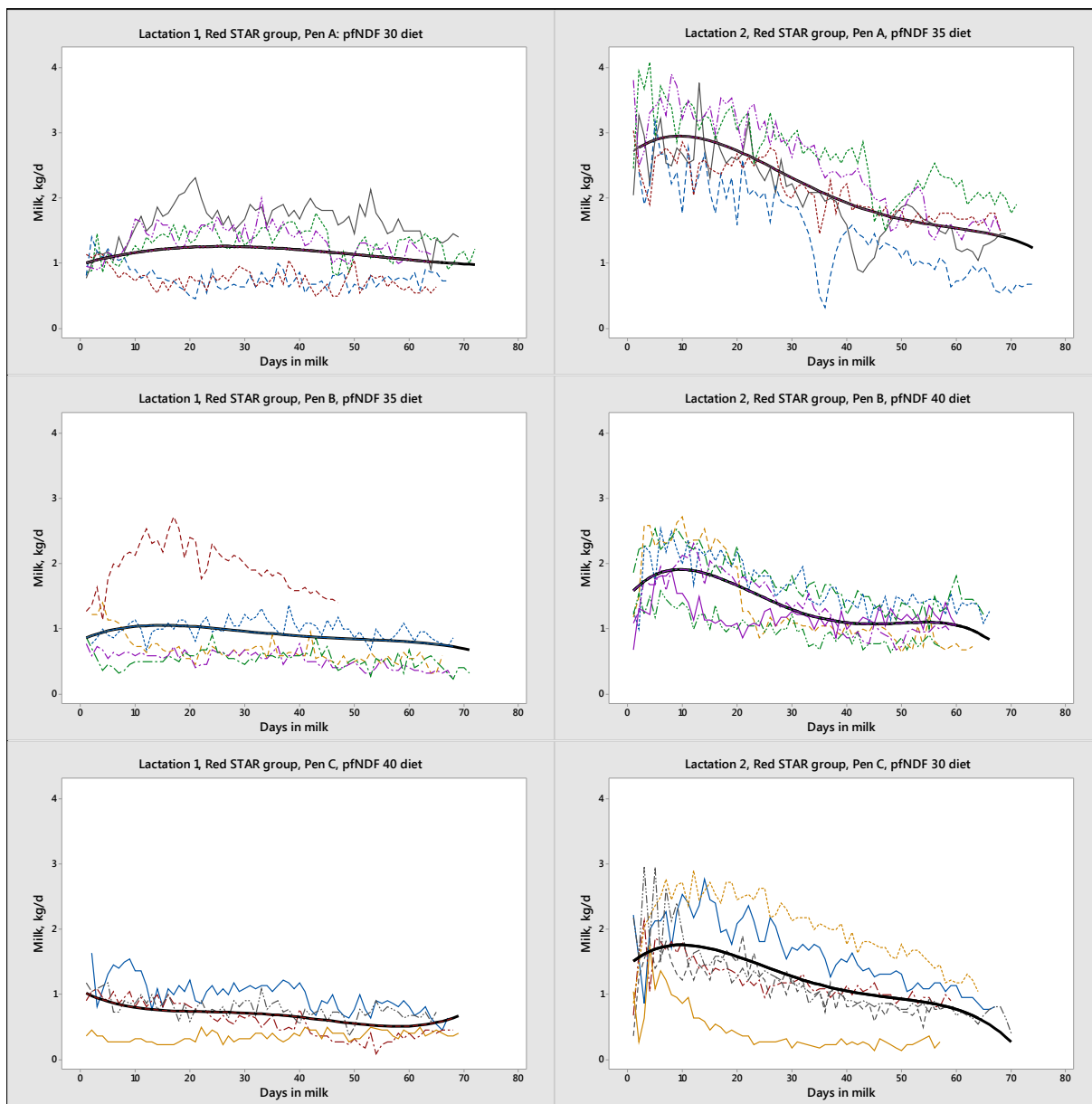


Figure 2. Individual ewe milk yields and fitted curves for diets with 30, 35, or 40% pfNDF in Red STAR group lactations 1 and 2.

### Conception, breeding

Successfully synchronized breeding allows for shortened lambing times. Of the 72-total lambings so far, an average of 82% occurred within the first half of the 30-day lambing season. This has substantial implications on labor and management because lambing periods could be limited to five 14-day periods per year. Further research is needed to investigate ideal dry and transition times for sheep milked on an accelerated

lambing system. Noteworthy is that rebreeding during lactation is feasible. This will have an impact on future management considerations.

Table 5. Conception rates.

Group and lactation	Ewes	Breeding start	Method	Scanned positive	Lambled	Parturition in first half of lambing period
Red STAR-1	18	6 Jun 2016	Teaser rams, CIDRs	14 (78%)	14	13 (93%)
Blue STAR-1	16	20 Aug 2016	Teaser rams, sponges	16 (100%)	16	11 (69%)
Green STAR-1	16	30 Oct 2016	Teaser rams	12 (75%)	12	11 (92%)
Red STAR-2	18	11 Jan 2017	Natural	17 (94%)	17	13 (76%)
Blue STAR-2	17	25 Mar 2017	Teaser rams, CIDRs in 13	13 (76%)	13	11 (85%)
Green STAR-2	18	6 June 2017	Teaser rams, CIDRs	17 (94%)	Lambing in October	

## Lambs

Lactation is highly impacted by fecundity (Peterson, 2005), with an increased potential of up to 63% for twin vs single lambs (Snowder and Glimp, 1991) and another 20% for triplets vs twins. The Cornell ewes achieved 3.43 delivered lambs per ewe within this experiment, allowing these ewes to make use of their fecundity potential. In comparison, 1.85 lambs per year for East Frisian, and 1.69 per year for Lacaune (Thomas, 2014) were recorded. This level of prolificacy has substantial impact on the economic viability of a sheep dairy farm. In addition to high milk yields, higher numbers of lambs can be sold for meat and breeding stock.

Literature suggests a lower body weight at 120 days for lambs reared artificially (McKusick et al., 2001), yet the lambs in this study have grown well (Table ). The death loss rate for lambs born alive has been very low at 1.4%. Further research might clarify the influence of dietary fiber levels on lamb vigor and growth potential.

Table 6. Growth of lambs.

STAR group and lactation	Number of lambs	Age, days	ADG, g/d
STARR-1	28	~50	263
STARB-1	34	~65	336
STARG-1	20	~45	269
STARR-2	36	~80	263
STARB-2	27	~20	290

## Conclusions and outlook

Milking sheep in short and frequent lactations has an impact on yearly milk yield and lactation length. Intensive selection and breeding is needed to make full use of the high milking potential of many ewes observed in this experiment. It is possible to breed sheep during lactation without a decline in fertility. This can be used to further investigate ideal dry and transition times to expand lactation length. It's necessary to supply ewes with high levels of nutrients during breeding. Dairy sheep genetics might be used to generate crossbreds to increase lactation persistency alongside lactation length for sheep milked in frequent lactations. The high lamb crop suggests possibilities to use crossbreed ewes not previously selected for dairy production for both lamb and milk production.

Traditional lambing times may need to be reevaluated. Ewes bred in March and lambing in August (STARB-2, n=13, results not shown) have a higher lactation yield than ewes bred in August and lambing in January (STARB-1, n=14). This concurs with European research (Sitzia et al., 2015), where ewes bred in June reached higher total lactation yields than ewes bred in autumn.

Future analyses will report measured dry matter and NDF digestibility and determine the inference of varying levels of pfNDF on VFA production in the rumen (Araujo, 2008) and on milk composition.

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# MAKING DECISIONS ABOUT NEW TECHNOLOGIES ON THE DAIRY

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## INTRODUCTION

Dairy producers and their advisors are confronted with a bewildering number of potential decisions or choices on a yearly, monthly, weekly, or even daily basis. These range from decisions relating to larger capital investments (e.g., a new milking center or conversion to automatic milking systems, a new transition cow barn, a new calf barn) to more modest capital investments (e.g., rumination tags, implementation of long day lighting, implementation of cooling in lactating and/or dry cows) to adoption of management practices (e.g., varying milking frequency, adjusting dry period length, adding an extra colostrum feeding or additional daily feeding to calves) to nutritional management decisions. Nutritional management decisions are also numerous and can relate to bigger picture decisions related to forage strategy or source, selection of dry and transition cow nutritional management strategies, or the decision to supplement specific nutrients or improved forms of nutrients or feed additives.

So, how can one sort through the decision-making process? My first recommendation is to develop a systematic process for decision-making. In the remainder of this paper I will attempt to outline elements that I think should be considered as parts of this decision-making process.

## ELEMENTS OF THE DECISIONMAKING PROCESS

What is the “Reward-Risk”?

This phase is conventionally termed “risk-reward”, but I have inverted this intentionally as I think that the conventional manner in which we express this tends to subconsciously focus one on the risk rather than the potential reward. In order to consider the “reward-risk” we should consider the statistical concepts that Dr. Dave Galligan and colleagues at the University of Pennsylvania applied to the types of decisions that we make in dairy management (Galligan et al., 1991). They defined Type I error as the risk of an economically unprofitable outcome following the decision to implement a management practice or use of a product and Type II error as the risk of loss of potential profit by failure to adopt a particular technology or management practice and gave examples of how they would apply these concepts in the decision of whether or not to feed sodium bicarbonate and whether or not to administer rbST. From summaries of research studies they determined mean responses and variation around them, determined break-even responses based upon input costs and output values, calculated the frequencies of all possible responses, and then modeled the economics associated with both types of error, with additional sensitivity analysis conducted based

upon variance for both input costs and output values. For these particular examples, they determined that the Type II economic risk far exceeded the Type I economic risk.

I think that it is important to consider that the importance of Type I vs. Type II error in decision-making depends upon the particular type of decision to be made that in turn relates largely to the size of the investment and/or magnitude of commitment. As an example, in the case of large capital investments such as new milking centers or conversion of a herd from conventional milking systems to automatic milking systems, Type I error risk is the larger concern because in these cases Type I errors may meaningfully decrease profitability and/or put the dairy farm business at risk. In my opinion, Type I error risk should also receive primary consideration in the decision for a farm to plant non-GE crops as described in Joe Lawrence's paper later in this conference, for significant pest or other crop system issues for which these technologies provide protection can have great impacts on overall profitability. For other management decisions (e.g., herd health and reproductive protocols, decisions to vary dry period length, nutritional strategies as will be discussed below), Type I errors are likely less of a problem and changes can be made relatively easily if it is determined that desired outcomes are not being achieved.

In general, I think that most decisions involving nutritional management are more at risk for Type II errors than Type I errors; however, my sense is that we generally focus more on the Type I risk than the Type II risk in these decisions. This is probably in large part because the input costs for adoption of a particular technology or nutritional strategy are easily determined and the specific response/value derived is often more challenging to determine, at least at the individual farm level given all of the other dynamic factors that can also influence outcomes of interest.

## CONSIDERATIONS FOR ADOPTION OF MANAGEMENT PRACTICES OR NUTRITIONAL STRATEGIES AND TECHNOLOGIES

Of course, if we apply a purely Type II-centric decision-making approach, we may adopt many management practices, nutritional strategies, and technologies – some of which will yield returns on the farm. There are a few things that I think should be considered when evaluating which adoption decisions to make.

### Biology and Potential Mode of Action

In my opinion, understanding the biology and potential mode of action is critical. Of course, some things are quite well understood (e.g., rbST, milking frequency, controlled energy strategies for dry cows, application of DCAD in dry cows, responses to AA) and other emerging or integrative areas are less well-understood (e.g., gut integrity/leaky gut, oxidative metabolism, immune/inflammatory mechanisms). This does not de-emphasize the importance of these latter areas, rather it is important to recognize that we are still defining these areas and how to best modulate them in a positive manner through management and/or nutrition.

## Develop a “Constellation of Evidence”

I think that decisions to adopt technologies have more certainty for success when responses are supported from various angles. Things that should be considered in the “constellation of evidence” are:

- Biology and potential mode of action (see above)
- Research and demonstration
  - Controlled, peer-reviewed University or Research Center work
  - Replicated (within dairy) commercial farm-based studies
  - Replicated (across dairies) commercial farm-based studies/demonstration
  - Meta-analytic approaches
- Practicality of implementation
- Experience

Controlled, peer-reviewed research should form the basis for determining potential responses and defining mode of action; however, this work can be complemented nicely with commercial-farm based studies or demonstration. Furthermore, meta-analytic techniques are being applied increasingly to areas (e.g., effects of monensin on metabolism – Duffield et al., 2008) in which there is a body of research that can be summarized using these approaches.

I would like to offer some “watch-out’s” relative to assessment of controlled studies, based upon nearly 25 years of experience and perspective gained over that timeframe doing these types of controlled studies in transition cow nutrition and management. First, many studies still are not well-replicated (we now strive for 25 to 30 cows per treatment in transition cow studies in which we are trying to make inferences relative to performance). As such, outcomes can be particularly influenced by decisions that the investigators make relative to cow removal prior to analysis because of adverse health events. There are several recent examples of studies in which either large numbers of cows were removed from the dataset or cows were selectively removed for the same disorder from some treatments but not others, with potentially important consequences for the results and conclusions of the studies. A second “watch-out” relates to performance of controls. We strive to have studies in which the control group performance is representative of how cows perform on well-managed commercial farms. There are recent studies in which the performance responses to treatment were very large, but the control group was clearly compromised for whatever reason and performing well below what might be expected on a commercial dairy. A third “watch-out” relates to the discussion of what is “biologically or economically significant” versus “statistical significance”. Again, in studies that have relatively low replication, a difference that would be considered very meaningful at the farm level may not be statistically significant. A good example of this relates to work that several groups, including our own, did a number of years ago looking at continuous lactation (zero dry period). Cows with zero days dry generally made 1.5 to 3 kg/d less milk in the subsequent lactation but differences were not statistically significant. Finally, are the



results internally consistent within study (i.e., do changes in blood chemistry and/or body weight and body condition score line up with the responses in milk yield, milk composition, and dry matter intake observed?).

### Projected Economic Returns

Of course, projected returns are a key consideration for adoption of technology. In addition to the approach that Galligan et al. (1991) illustrated, partial budget analysis to include changes in revenue or other benefits and changes in investment or inputs to determine marginal returns can be conducted. Within each of these, clear conveyance of the anticipated changes, consideration of whether the numbers and assumptions are hard or soft (level of certainty) is important. Furthermore, sensitivity of the final outcome to variation in response or changes in the input costs/output value should be evaluated.

### Managing Expectations

Not every decision is going to be a home run, or even a hit. If there is heterogeneity in responses across studies on a topic, it should be represented in some manner. In my opinion, this helps to build credibility and take focus away from specific responses observed in an individual study and put it more on the pattern of responses seen across multiple studies.

### What is the “Opportunity Cost” for Labor and Management?

Owners and managers on-farm as well as their advisors should keep in mind the opportunity cost for labor and management relative to adoption of management practices or technologies. This consideration will help to prioritize and keep focus on whether there are other management practices that would yield more potential return for the effort required to implement.

### TWO EXAMPLES – INCREASED MILKING FREQUENCY AND MONITORING AND TREATMENT OF HYPERKETONEMIA

#### Assessment of 4X/2X Milking Strategies

A number of studies over the past 20 years have focused on increased milking frequency (IMF) of cows during the first 21 or so days postcalving. Following the original work in Israel in which cows milked 6X for the first 42 d postcalving maintained milk yields about 5 kg/d higher after return to 3X milking compared with cows milked 3X starting at calving, several studies evaluated 4X/2X milking schemes (fresh cows were generally milked first and again last in a 2X milking schedule) in University herds and on commercial dairy farms. These studies generally demonstrated carryover responses ranging from 2 to 4 kg/d of milk (Hale et al., 2003; Fernandez et al., 2004; Dahl et al., 2004; Wall and McFadden, 2007; Soberon et al., 2010; Soberon et al., 2011). The only study that demonstrated negative production responses to IMF was that of Van Baale et al. (2005) involving 6X/3X milking; however, they determined that on the large

commercial dairy in which they conducted the research, cows milked 6X were away from the fresh pen for milking more than 6 h per day, which likely had severely negative impacts on time budgets of those cows. Further, in our commercial farm-based work (Soberon et al., 2011), in general, the farms that had better management of stocking densities in the fresh pen and better transition management overall had better responses to IMF, although all farms in that dataset had a positive response to IMF.

Partial budget analysis suggested increased net revenue of about \$80 per cow for adoption of 4X/2X milking, with most of the increased input cost associated with the increased feed requirements to support the additional milk yield. Changes in feed cost would be directly proportional to responses. Despite this, there has been very little adoption of this management practice on dairies milking 2X. Perhaps this is a perception of opportunity cost of labor and management, lack of confidence of seeing a response despite the available information, or simply lack of willingness to adopt a practice that will increase owner/manager labor on many of these dairies.

### Monitoring and Treatment of Hyperketonemia

Following commercial-farm based research that established associations between cow- and herd-level prevalence of hyperketonemia (subclinical ketosis) and increased incidence of clinical disease, decreased milk yield, and impaired reproductive performance (Ospina et al., 2010a; 2010b, 2010c), McArt and coworkers conducted studies on four farms in New York and Wisconsin to determine the epidemiology of subclinical ketosis as well as the outcomes of intensive testing and treatment strategies using handheld BHBA meters and propylene glycol drench as a treatment regimen (McArt et al., 2011; 2012a; 2012b). They demonstrated increased milk yield, decreased DA, decreased herd removal, and increased first service conception rate for cows detected and treated with propylene glycol. Further economic analysis of varying intensities of testing with associated propylene glycol treatment yielded net returns ranging from \$7 to \$11 per cow for herds with 40% incidence (~ 2X prevalence) and greater returns for herd with higher prevalence and incidence. Although the economic returns of intensive diagnostics and associated treatment are favorable, there is likely an opportunity cost of management and labor that should be considered. At a minimum, schemes such as the one in Figure 1 that Ospina et al. (2013) proposed to monitor prevalence with management decision-making based upon prevalence can be very effective ways to monitor and manage hyperketonemia in a targeted manner.

## SUMMARY AND CONCLUSIONS

Decision-making at the herd level can be complex for both dairy producers and their advisors. Having a process that is systematic and that considers multiple aspects and implications of decisions can lead to better decision-making overall. Producers and their advisors should weigh the “reward-risk” of decisions, and not let concern about making Type I errors result in greater losses through Type II errors related to failure to adopt technologies or management practices. Developing a “constellation of evidence” consisting of biological mode of action, research and demonstration to support,

likelihood of effective implementation, and experience can help to determine which adoption decisions to make, keeping in mind management systems, practicality, and opportunity cost for labor and management.

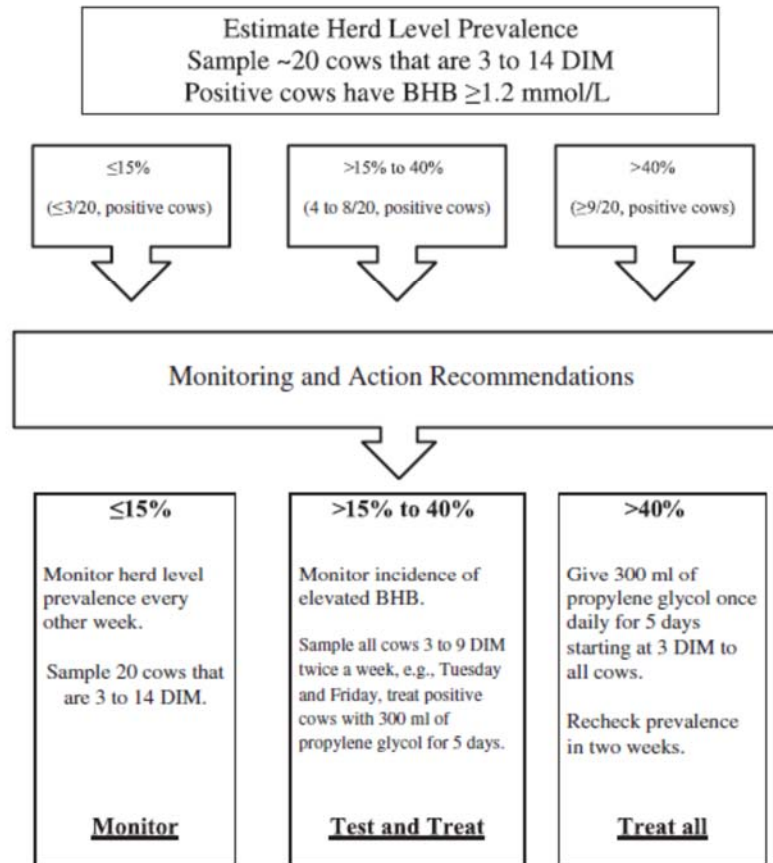


Figure 1. Testing scheme for prevalence of hyperketonemia in fresh cows with associated recommendations for monitoring and treatment. From Ospina et al., 2013.

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# **EFFECT OF DIETARY SUPPLEMENTATION OF TWO FORMS OF A B-VITAMIN AND CHOLINE BLEND ON THE PERFORMANCE OF HOLSTEIN CALVES DURING THE TRANSITION AND EARLY POSTWEANING PERIOD**

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## **INTRODUCTION**

An optimal dairy replacement raising system is crucial for optimizing milk production and profit of dairy farms. The replacement system should be developed with the objective of providing targeted management and nutrition to dairy heifers during the entire course of development to meet specific and objective goals. The transition from liquid to solid diets during weaning is often an impediment for maintaining nutrient balance and growth (Weary et al., 2008) while starter formulation and nutrient content are important during this phase. Postweaning growth needs to be optimized to take advantage of the high efficiencies of growth and lower cost per unit of weight gain that can be attained and thus attention should be given to strategies for maximizing the performance through the transition and postweaning period (Kertz et al., 1998).

Weaning is a critical process that dictates significant anatomic and physiologic adaptations to facilitate appropriate solid feed intake, ruminal function and post-absorptive utilization of the fermentation end-products (Baldwin et al., 2004). Thus, the weaning period should be approached as an opportunity to adequately prepare dairy heifers to face the ruminant state while capitalizing on the benefits of enhanced growth. From a nutritional standpoint, weaning could be facilitated by the provision of a palatable diet with a nutrient profile that would enable proper ruminal fermentation for rapid development at low levels of intake, and supply the calf with an adequate profile of post-ruminally available nutrients to maintain expected growth rates.

Supplementing B-vitamins and choline to calves' diets is a consideration as they facilitate metabolic processes by acting as coenzyme factors or providing and transferring methyl-groups (McDowell, 2000), which are necessary for many metabolic functions related to health and growth. The NRC (2001) does not recommend supplementation of these nutrients to the dry feed for dairy calves under the assumption that rumen synthesis provides them in sufficient amounts after weaning. This assumption has not adequately been tested in calves with higher targeted growth rates. Nevertheless, even in fully developed ruminants, the supplementation of these vitamins has been demonstrated to improve health and performance (Girard and Matte, 2005, Lean and Rabiee, 2011). This suggests that demand for these nutrients during physiological stages of stress and high metabolic activity might exceed the dietary supply and ruminal synthesis. Before weaning, milk or milk replacer provide sufficient quantities of these vitamins to the young calf, but this supply is reduced over weaning (Waugh et al., 1947, Girard et al., 1989), while ruminal activity and microbial vitamin synthesis are still not fully developed (McDowell,

2000). During this period of decreased dietary supply, calves have a significant metabolic demand due to their lean growth and as part of change in metabolism to the ruminant state.

Research in this area is limited, however, there is evidence suggesting that B-vitamin supply during and after weaning could be insufficient to support optimal growth in dairy calves (Dumoulin et al., 1991, Girard and Matte, 1997). Although B-vitamins and choline could be added to starter feeds, the potential degradation of these vitamins by ruminal microbes might restrict the benefit of their supplementation (Santschi et al., 2005). Alternatively, rumen-protected forms of B-vitamins and choline have been used with considerable success in mature ruminants (Sacadura et al., 2008).

This study was conducted to evaluate the effect of combined supplementation of B-vitamins and choline, in non-protected and rumen-protected forms, during the transition and post-weaning period on performance of dairy calves fed a diet balanced for all nutrients, including all essential amino acids (EAA) and to achieve at least 1 kg/d gain. Our hypothesis was that these vitamins are limiting for optimal calf performance during the transition phase and, to be effective, they must be fed in a rumen-protected form.

## METHODOLOGY

All protocols involving animals were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee. Sixty-one Holstein calves (37 female and 24 male) born at the Cornell University Ruminant Center (Harford, NY) were enrolled. Within the first two hours of life, calves received two doses of colostrum extract (Immu-Prime; Sterling Technology, Brookings, SD) and were fed 4 L of colostrum replacer (240 g globulin proteins, Nursemate Plus 150; Sterling Technology, Brookings, SD). Twelve hours after birth, 2 L of pooled colostrum ( $\geq 22$  on the Brix scale) were fed. Subsequently, calves were moved to a naturally ventilated calf barn, measured for BW ( $42.9 \pm 0.8$  kg) and height ( $76.5 \pm 0.5$  cm at withers and  $80.2 \pm 0.5$  cm at hip), and housed in individual pens until 13 wk of age.

Calves were offered milk replacer (Excelerate; Milk Specialties Co., Eden Prairie, MN; Table 1) starting at a feeding rate of 0.85 kg DM/d and increasing progressively over the first 21 d up to 1.6 kg DM/d. At 49 d of age, the weaning process was started by withdrawing 0.1 kg DM/d until d 63 when weaning was completed. Milk replacer was reconstituted at 15% solids and offered at 39 °C three times per day with nursing bottles containing 3.8L of replacer. A textured calf starter, specifically formulated for this study (Table 1), was offered ad libitum from wk 4 to 13. For adequate interpretation of the B-vitamin supplementation, the starter did not contain any added B-vitamins or choline and was formulated to meet all EAA requirements, based on body composition data (Van Amburgh et al., 2015) and the CNCPS v.7 (Higgs and Van Amburgh, 2016) predictions of amino acid supply, to ensure that they were not first limiting, since the metabolism of these nutrients is tightly interrelated (McDowell, 2000, Girard and Matte, 2006). The starter was pelleted at a commercial feed mill (Purina Animal Nutrition, Erwin, NY) and blended at Lutz Feeds (Oneonta, NY). Fresh water was available ad libitum.

At wk 3, calves were assigned to one of the three treatments in a randomized design. Treatments were as follows: a rumen protected B-vitamin and choline blend (**RPBV**, n = 20), a 70:30 mix of fat concentrate and non-protected B-vitamin and choline blend (**UPBV**, n = 22) and an unsupplemented group receiving a fat concentrate as a placebo (**CTRL**, n = 19; Equi-Calorie 100, Jefe Nutrition Inc., Saint-Hyacinthe, QC, Canada). The fat concentrate was included in UPBV and CTRL treatments to keep diets isocaloric.

Table 1. Ingredient of the formulated starter, and chemical composition and vitamin concentration of milk replacer and starter fed.

Ingredient composition (% of DM)	Starter	Item	Milk replacer	Starter <sup>1</sup>
		DM, %	94.7	86.6
Pellet		Chemical composition (% of DM) <sup>2</sup>		
Treated soybean meal	19.8	Crude protein	28.5	25.5
Wheat middlings	19.3	Crude fat	15	2.96
Canola meal solvent	6.6	aNDFom	-	25.1
Dextrose	3.3	Ash	6.82	8.18
Dried whey	5.9	Calcium	1	0.96
Blood meal	4.0	Phosphorus	0.71	0.63
Methionine analog	0.7	Cobalt, mg/kg	1.22	1.4
Minerals	1.8	Vitamin A, IU/kg <sup>3</sup>	16,530	7,273
Fat	0.7	Vitamin D, IU/kg <sup>3</sup>	5,510	2,424
Vitamin ADE premix	0.3	Vitamin E, IU/kg <sup>3</sup>	110.2	29.9
Monensin	0.03	ME, Mcal/kg	4.6	2.5
Flavor/odor enhancer	0.1	B-Vitamin concentration (mg/kg DM)		
Pellet Binder	1.0	Thiamin	17.3	3.9
Flaked corn	20.1	Riboflavin	29.9	5.3
Beet pulp shreds	13.2	Niacin	152	75.6
Molasses	3.3	Pantothenic acid	91.7	14.4
		Pyridoxine	9.36	4.19
		Biotin	0.85	0.57
		Folates	1.04	0.89
		Vitamin B <sub>12</sub>	0.096	0.005
		Choline, mg/kg DM	1,600	1,260

<sup>1</sup>Amino acid content (% Nitrogen): 7.1% Asp, 3.2% Thr, 4.6% Ser, 9.7% Glu, 3.6% Pro, 5.2% Gly, 5.3% Ala, 4.3% Val, 2.2% Cys, 2.1% Met, 2.6% Ile, 6.5% Leu, 2.6% Try, 3.4% Phe, 3.0% His, 7.1% Lys, 4.3% Trp, 12.2% Arg.

<sup>2</sup>As reported by the manufacturer for milk replacer, and as measured for the starter grain

<sup>3</sup>As reported by the manufacturer for milk replacer, and as formulated for the starter grain

Vitamin blends were formulated to contain all B-vitamins (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folate and vitamin B<sub>12</sub>) and choline considering the estimated weaned calf requirements. This estimation considered whole bovine milk vitamin concentrations (Davis and Drackley, 1998), reported requirements for swine (NRC, 2012) and ruminants (Marston, 1970, CSIRO, 2007) and studies reporting productive benefits with B-vitamin supplementation in dairy cattle (Dumoulin et al., 1991, Lévesque et al., 1993, Graulet et al., 2007). The B-vitamin and choline blends were mixed



and manufactured by Jefe Nutrition Inc. (Saint-Hyacinthe, QC, Canada). To ensure consumption and adequate dosing, treatments were weighed into gelatin capsules and administered orally once a day using a balling gun, based on the previous day starter intake. Choline chloride was mixed with the B-vitamin blend right before assembling the UPVB capsules. Vitamin treatments and placebo were fed at  $0.39 \pm 0.001\%$  and  $0.28 \pm 0.001\%$  of the starter intake, respectively.

Body weight and height were measured weekly. Milk replacer and starter intake were recorded daily. Blood was collected weekly from wk 3 to 13 for measurement of B-vitamin status and plasma urea nitrogen (PUN) and BHB. Samples of milk replacer, starter and supplements were sent to commercial labs for chemical analysis (Cumberland Valley Analytical Services, Maugansville, MD) and B-vitamins and choline analysis (Covance Inc., Princeton, NJ). For amino acid content determination, a starter sample was ground to 1 mm and analysed by HPLC following hydrolysis at  $110^{\circ}\text{C}$  in a block heater for 21 and 168 h for Trp and the rest of the amino acids, correspondingly, following the procedure described by (Fessenden et al., 2017). Prior to the beginning of the study, the unavailable nitrogen ( $34.97 \pm 0.61\%$ ) in the RPBV was determined in duplicates according to the in vitro indigestibility assay described by (Ross et al., 2013). In a separated analysis, in vitro rumen nitrogen stability of the RPBV was determined to be  $82.73 \pm 2.00\%$ , after 18 h of fermentation.

The PUN and BHB concentrations were measured using enzymatic colorimetric assays based on commercial kits (No. 640; Sigma-Aldrich, St. Louis, MO; and  $\beta$ -Hydroxybutyrate Liquicolor; Stambio Laboratory, Boerne, TX; respectively). Plasma urea nitrogen and BHB were measured from wk 5 to 13. Folates and vitamin B<sub>12</sub> were determined for samples taken at wk 3, 7, 9 and 13 by radioassay using a commercial kit (Simultrac B<sub>12</sub>/ Folate-S; MP Biomedicals, Santa Ana, CA). Plasma measurements were adjusted to 35% hematocrit, to account for any variation in hydration status.

The data were organized weekly and by periods relative to weaning and were defined as follows: preweaning (wk 4 to 7), weaning (wk 8 and 9) and postweaning (wk 10 to 13). Variables for which the change over time were studied (blood parameters, BW and height) were analyzed as a completely randomized design with a mixed-effects model including the fixed effects of treatment, week and their interaction and a random effect of calf. Measurements taken at wk 3, were used as covariates for the analysis of growth traits. A fixed effects model including the effect of treatment only was used to analyze outcomes by period or at wk 3 only (weight and height ADG, DMI, ME intake, B-vitamin and choline intake, and feed efficiency). Analysis were performed using R (v. 3.3.2, R Core Team, 2016). Pairwise comparisons were done by week or by period, using a Tukey test to correct for multiple comparisons using the “lsmeans” in R. All reported mean values are arithmetic means and standard error as parameter of variation. Significance was declared at  $P \leq 0.05$  and trends were stated at  $0.05 < P \leq 0.10$ .

## RESULTS AND DISCUSSION

### Dry Matter and Nutrient Intake

Intake of milk replacer was similar among groups at wk 3, before treatments were administered ( $1.12 \pm 0.02$  kg DM/d,  $P = 0.87$ ). During the experimental period, no differences were observed among treatments for intake of milk replacer, starter, total DM or ME ( $P \geq 0.35$ , Figure 1A). Calves consumed less milk replacer than what was offered to them, indicating that they were not feed restricted under the management conditions of the study. Other researchers have similarly observed no effects on DMI, before and during weaning, when supplementing a rumen-protected B-vitamin and choline blend in the diet (Wood et al., 2016) or folic acid parenterally (Dumoulin et al., 1991). Dumoulin et al. (1991) did not observe differences in the response to vitamin supplementation between calves fed restricted or ad libitum. However, they observed that calves under non-restricted feeding increased their intake of concentrate after weaning when receiving supplemental folic acid.

Crude protein intake followed the same pattern as total DMI (data not shown), as the protein content was not remarkably different among feeds. Preweaning intake of most B-vitamins and choline did not differ among treatments ( $P > 0.73$ ), except for folates and vitamin B<sub>12</sub>, for which supplemented groups showed higher intakes ( $P < 0.01$ , Table 2). Following the reduction in milk replacer intake due to weaning, estimated intake of B-vitamins and choline decreased for the CTRL group, while supplemented calves had higher intakes for most of these vitamins ( $P \leq 0.03$ ). As the starter intake of calves increased after weaning, the calculated intake of all vitamins, except vitamin B<sub>12</sub>, increased for CTRL fed calves. The calves assigned to UPBV and RPBV treatments had higher intakes of all B-vitamins and choline than the unsupplemented calves after weaning ( $P < 0.001$ ). For most B-vitamins and choline, all treatments reached similar or greater intakes pre- compared to post-weaning.

### Folates and Vitamin B<sub>12</sub> plasma concentrations

Unlike simple-stomached species, B-vitamins and choline consumed by ruminants in the diet and in supplements cannot be considered as the net supply because of alteration, utilization and synthesis of these nutrients by rumen microbes. Thus, to evaluate the net vitamin supply, circulating vitamin levels are more useful (Girard and Matte, 1988, 1997). Only plasma folates and vitamin B<sub>12</sub> levels were measured to evaluate the effect and form of supplementation and the data in Figure 1B illustrates the plasma concentrations for both vitamins.

From the initial measurement at wk 3 ( $5.37 \pm 0.19$  ng/mL), plasma folates increased dramatically throughout the study reaching  $12.20 \pm 0.26$  ng/mL at wk 13 regardless of treatment ( $P > 0.14$ ). The increase observed with age corresponds to the progression of ruminal function and folate synthesis by the rumen microflora. Dumoulin et al. (1991) observed higher blood folate levels in ruminant calves fed ad libitum compared to restricted-fed calves despite similar intake of folates, indicating that the

greater DM being digested ruminally could have explained the differences in folate status. Recent work has corroborated the positive apparent ruminal synthesis of folates in mature ruminants (Santschi et al., 2005, Castagnino et al., 2016). Calves in the current study had the same DMI among treatments, but supplemented calves consumed more folates than CTRL group during the entire experiment. The efficacy of the non-protected folic acid provided to UPBV calves was probably diminished by the ruminal activity. In dairy cows, it has been estimated that folate ruminal disappearance is 97% (Santschi et al., 2005).

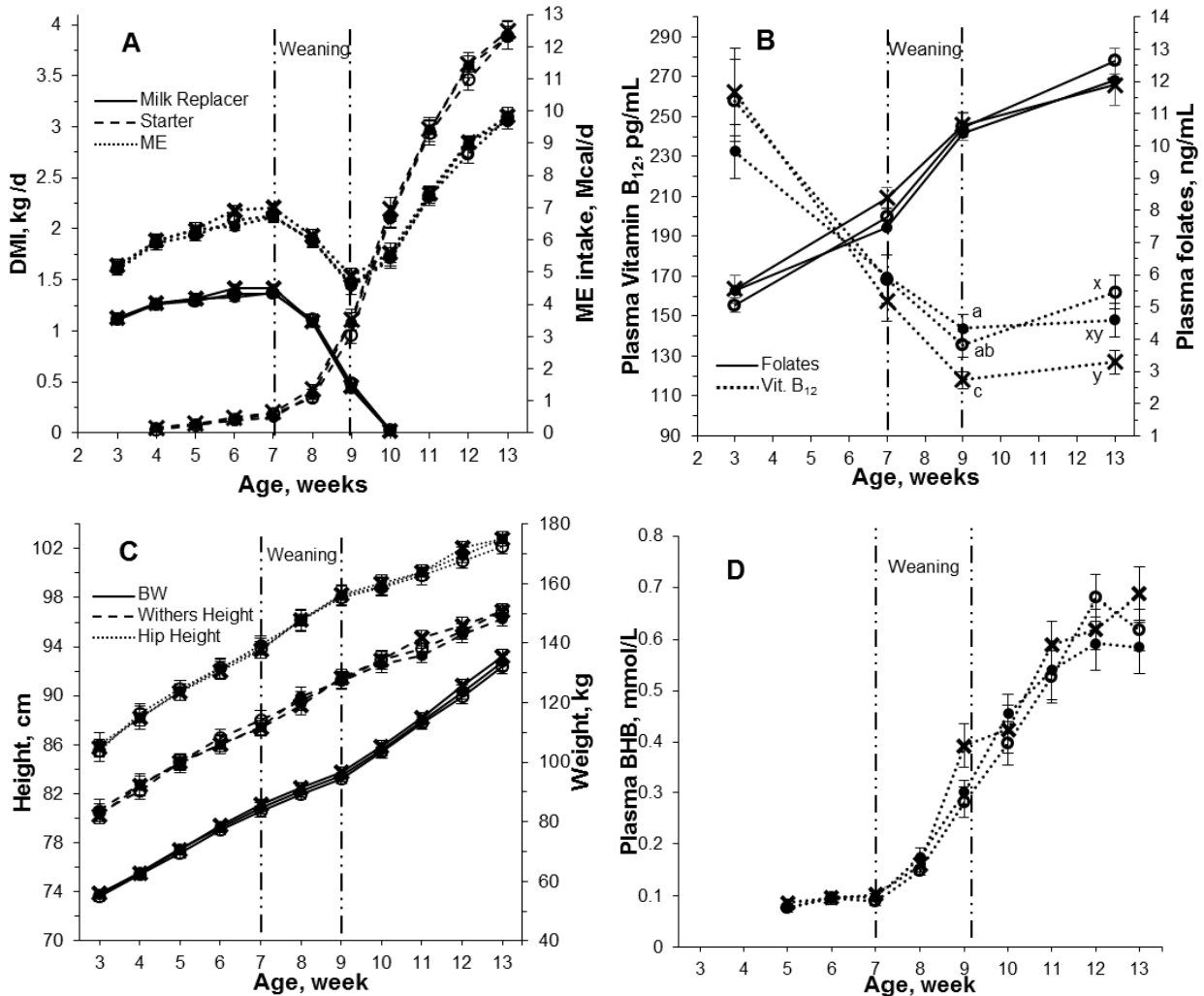


Figure 1. Mean DMI of milk replacer and starter and mean estimated total ME intake (A), plasma levels of folates and vitamin B<sub>12</sub> (B), body weight, withers height and hip height (C) and plasma BHB (D) of calves supplemented with a rumen-protected B-vitamin and choline blend (●), a non-rumen protected B-vitamin and choline blend (○) or a placebo (X) from 4 to 16 wk of life. Vertical bars represent SE. a and b differ P < 0.10; x and y differ P < 0.05.

Table 2. Means of dry matter, metabolizable energy, B-vitamins and choline intake, average daily gain, and feed efficiency for calves supplemented with a placebo (CTRL), a non-rumen protected B-vitamin and choline blend (UPBV), or a rumen-protected B-vitamin and choline blend (RPBV) at periods relative to weaning.

Item <sup>2</sup>	Treatment <sup>1</sup>			SE <sup>3</sup>
	CTRL	UPBV	RPBV	
<b>Preweaning</b>				
DMI, kg/d <sup>4</sup>	1.48	1.43	1.43	0.02
ME intake, Mcal/d	6.55	6.34	6.35	0.11
Thiamin intake, mg/d	23.94	23.47	23.56	0.41
Riboflavin intake, mg/d	41.20	40.83	40.72	0.71
Niacin intake, mg/d	215.56	211.53	211.56	3.77
Pantothenic acid intake, mg/d	126.12	125.65	125.56	2.56
Pyridoxine intake, mg/d	13.21	13.47	13.53	0.24
Biotin intake, mg/d	1.22	1.20	1.20	0.02
Folate intake, mg/d	1.52 <sup>b</sup>	1.72 <sup>a</sup>	1.70 <sup>a</sup>	0.04
Vitamin B <sub>12</sub> intake, ng/d	130.80 <sup>b</sup>	175.50 <sup>a</sup>	177.70 <sup>a</sup>	4.26
Choline intake, mg/d	2,327.43	2,301.22	2,303.68	42.09
ADG, kg/d	1.09	1.02	1.03	0.02
Feed efficiency	0.73	0.72	0.72	0.01
<b>Weaning</b>				
DMI, kg/d <sup>4</sup>	1.59	1.49	1.51	0.03
ME intake, Mcal/d	5.46	5.31	5.27	0.14
Thiamin intake, mg/d	16.42	17.37	17.27	0.62
Riboflavin intake, mg/d	27.27 <sup>b</sup>	31.20 <sup>a</sup>	29.69 <sup>ab</sup>	1.08
Niacin intake, mg/d	176.16	183.68	181.66	4.43
Pantothenic acid intake, mg/d	82.19 <sup>b</sup>	97.50 <sup>a</sup>	94.40 <sup>a</sup>	3.31
Pyridoxine intake, mg/d	10.48 <sup>b</sup>	13.45 <sup>a</sup>	13.79 <sup>a</sup>	0.35
Biotin intake, mg/d	1.10	1.15	1.14	0.02
Folate intake, mg/d	1.40 <sup>b</sup>	2.62 <sup>a</sup>	2.64 <sup>a</sup>	0.12
Vitamin B <sub>12</sub> intake, ng/d	78.30 <sup>b</sup>	316.40 <sup>a</sup>	343.70 <sup>a</sup>	20.90
Choline intake, mg/d	2,212.48	2,356.55	2,383.41	65.29
ADG, kg/d	0.78	0.79	0.79	0.03
Feed efficiency	0.49	0.53	0.53	0.01
<b>Postweaning</b>				
Starter intake, kg/d	3.17	3.10	3.14	0.06
ME intake, Mcal/d	7.98	7.82	7.86	0.15
Thiamin intake, mg/d	12.51 <sup>b</sup>	17.10 <sup>a</sup>	18.66 <sup>a</sup>	0.63
Riboflavin intake, mg/d	17.05 <sup>c</sup>	35.42 <sup>a</sup>	31.68 <sup>b</sup>	1.41
Niacin intake, mg/d	241.45 <sup>b</sup>	299.16 <sup>a</sup>	297.26 <sup>a</sup>	10.02
Pantothenic acid intake, mg/d	46.41 <sup>b</sup>	118.03 <sup>a</sup>	112.72 <sup>a</sup>	5.29
Pyridoxine intake, mg/d	13.99 <sup>b</sup>	28.93 <sup>a</sup>	30.34 <sup>a</sup>	1.30
Biotin intake, mg/d	1.81 <sup>b</sup>	2.26 <sup>a</sup>	2.20 <sup>a</sup>	0.08
Folate intake, mg/d	2.46 <sup>b</sup>	8.66 <sup>a</sup>	8.40 <sup>a</sup>	0.12
Vitamin B <sub>12</sub> intake, ng/d	16.50 <sup>b</sup>	1,171.60 <sup>a</sup>	1,246.80 <sup>a</sup>	78.79
Choline intake, mg/d	1,018.02 <sup>b</sup>	5,169.12 <sup>a</sup>	5,249.43 <sup>a</sup>	179.05
ADG, kg/d	1.40	1.33	1.36	0.02
Feed efficiency	0.44	0.43	0.43	0.01

<sup>a,b,c</sup>Means within a row without a common superscript differ ( $P < 0.05$ )

<sup>1</sup>CTRL, n = 19; UPBV, n = 22; and RPBV, n = 20.

<sup>2</sup>Preweaning = weeks 4 to 7; Weaning = Weeks 8 and 9; Postweaning = Weeks 10 to 13. Data presented as arithmetic means

<sup>3</sup>Overall SE is shown

<sup>4</sup>DMI includes milk replacer and starter intake

However, the lack of response of plasma folates to the additional folic acid provided with the RPBV treatment, suggests that, 1) the vitamin could have been rapidly cleared preventing its accumulation in plasma, 2) it was released from the matrix at a different level of the intestine than the proximal duodenum and jejunum, where its absorption takes place (Santschi et al., 2005), 3) the quantity of folate synthesized ruminally diminished the importance of the supplemental folic acid, or 4) a combination of all of these factors took place.

Plasma B<sub>12</sub> decreased 34% during the preweaning period, independent of treatment ( $P > 0.96$ ). By the end of weaning (wk 9) vitamin B<sub>12</sub> continued decreasing with RPBV ( $143.91 \pm 6.9$  pg/mL) calves tending to have higher levels than CTRL ( $117.98 \pm 4.27$  pg/mL;  $P = 0.09$ ), but no differences were detected between UPBV calves and the other group ( $135.8 \pm 6.37$  pg/L;  $P > 0.23$ ). After weaning, plasma concentrations of vitamin B<sub>12</sub> stabilized with UPBV calves having higher values ( $162.01 \pm 8.03$  pg/mL) than CTRL calves ( $126.94 \pm 5.82$  pg/mL;  $P = 0.02$ ), while RPBV group had intermediate levels ( $148 \pm 8.64$  pg/mL;  $P > 0.24$ ). When only evaluating the plasma concentrations of vitamin B<sub>12</sub>, both forms of vitamin supplements showed the same effectiveness to supply vitamin B<sub>12</sub>, thus appreciable quantities of the non-protected cyanocobalamin could potentially have reached the intestine. In previous work in adult ruminants, the ruminal disappearance of this vitamin (63%) is intermediate (Santschi et al., 2005). This suggests that if the ruminal stability and intestinal availability measured for the rumen-protected supplement are considered, RPBV calves would have received an equivalent amount of cyanocobalamin at the intestinal level as the UPBV calves. Thus, these results suggest that the ruminal use and degradation of B-vitamins described in mature ruminants occur in similar magnitude in the developing ruminant. In a similar way, they prove that, at least for cyanocobalamin, the technology of physical protection successfully prevents ruminal degradation and allows intestinal absorption.

Vitamin B<sub>12</sub> plasma levels and pattern over time coincides with what has been characterized in heifers raised on milk and concentrates before weaning and fed on pasture thereafter; decreasing from 160 to 77 pg/mL from 17 to 198 days of age, and stabilizing at 130 pg/mL at 342 d (Grace et al., 2014). Plasma concentrations observed in the present study were higher during the preweaning period which could be attributed to the greater amount of liquid diet offered and the higher concentration of the vitamin in the milk replacer. The decrease in plasma vitamin B<sub>12</sub> concentration with age parallels the estimated intake of the vitamin and liquid feed in the CTRL group. Serum concentrations of this vitamin in veal calves behaved differently, increasing during the first weeks of life and stabilizing at approximately 422.6 pg/mL (Girard and Matte, 1988). This observation, when linked to the vitamin B<sub>12</sub> concentration in plasma while consumption of milk replacer was still elevated suggests that calves might have increased their demand for this vitamin. It is likely that the metabolic activity occurring during the ruminant state started with the intake of dry feed and augmented the demand for this vitamin even before weaning was initiated. Vitamin B<sub>12</sub> serves as coenzyme of methylmanolonyl-CoA mutase, an enzyme essential for the integration of propionate into the Krebs cycle and its use as gluconeogenic substrate; consequently, the demand of this vitamin by ruminants is about 10 times the oral requirement for simple stomached species (McDowell, 2000). Thus, the decrease of this vitamin before and during weaning suggests that metabolic adaptations

to the ruminant state might occur before calves are able to increase their ingestion of solid feed.

Ruminant animals can obtain vitamin B<sub>12</sub> from ruminal synthesis. The apparent ruminal synthesis of vitamin B<sub>12</sub> is positive in dairy cows (Castagnino et al., 2016) and seems to be an important source of the vitamin for those animals. To be able to carry out this synthesis, rumen microflora needs an adequate dietary supply of cobalt; the established dietary cobalt requirement for ruminants is 0.11 mg/kg of diet (NRC, 2001). The starter offered in our study should have provided sufficient cobalt to support ruminal vitamin B<sub>12</sub> synthesis. Other factors that appear to affect the ruminal synthesis of this vitamin include the acidogenic capacity of the diet, being negatively associated with starch fermentability and positively correlated with fiber intake (Sutton and Elliot, 1972). Since plasma vitamin B<sub>12</sub> concentrations after weaning were in parallel with the values observed in grazing heifers (Grace et al., 2014), it could be implied that ruminal conditions were favorable despite calves being fed exclusively the formulated starter. The quantity and quality of the fiber in the current starter might have enabled adequate fermentation even with the considerable levels of starch (17.8% DM) and sugars (14.1% DM). Khan et al. (2011a) found better solid feed intake, greater rumen development and higher ruminal pH by feeding forage to calves fed high volumes of milk and a calf starter. When both the grass hay and starter DMI and NDF content (18.6 and 62.4% DM, respectively) were considered, an integrated NDF content of the diet of 29.7% DM was estimated. The fiber content of the starter used in our study was not far from this value. In addition, the fibrous ingredients used in the formulation (wheat middlings and beet pulp) are characterized by containing appreciable amounts of soluble fiber and their aNDFom by having a greater potentially digestible fraction and a faster rate of digestion than most forages (Raffrenato and Van Amburgh, 2010, Zontini, 2016). These starter characteristics might have benefited DMI and ruminal pH. The estimated soluble fiber content of the starter was 6.36% DM.

### Growth and Feed Efficiency

Although circulating concentrations of B-vitamins and choline might help to evaluate vitamin status and supplementation effectiveness, performance should serve as better criteria to determine adequacy of these vitamins. Body weight ( $55.58 \pm 0.89$  kg), wither height ( $80.52 \pm 0.40$  cm), hip height ( $85.76 \pm 0.44$  cm) and their respective rates of gain ( $0.83 \pm 0.05$  kg/d,  $0.20 \pm 0.01$  cm/d and  $0.19 \pm 0.04$  cm/d) did not differ among groups at wk 3 ( $P > 0.20$ ). BW increased with age ( $P < 0.001$ ) but no differences were detected among treatments during the entire period ( $P = 0.64$ ; figure 1C). Birth weight was doubled before weaning started and tripled by the end of the experiment.

Overall ADG was  $0.99 \pm 0.01$  kg/d over the entire experiment and differed by period. Average daily gain was  $1.05 \pm 0.02$  kg/d before weaning, diminished to  $0.78 \pm 0.03$  kg/d during weaning and rapidly increased to  $1.36 \pm 0.02$  kg/d after weaning. These rates of gain were not affected by treatments ( $P > 0.45$ ; Table 2). These ADG are consistent with growth rates obtained in other experiments providing adequate nutrients and feed availability to dairy calves (Khan et al., 2011b, Eckert et al., 2015). The slower

ADG observed during weaning is tied with the reduction in ME intake. This reduction in the rate of growth with weaning is commonly seen in the literature. However, experiments performed under a high level of nutrition have reported a more exacerbated growth slump during weaning (Terré et al., 2007) and the week after (Terré et al., 2006a, Stamey et al., 2012). This discrepancy might be attributed to differences in weaning management since in these studies calves were weaned earlier, during a shorter period and less gradually than in our study. Eckert et al. (2015) reported a similar growth reduction during the week of weaning (about 20% of preweaning ADG) of calves weaned at 8 wk of age in a step-down manner. Although in the present experiment weaning was started at the same age and performed gradually for a longer period, calves demonstrated this reduction in ADG during the two weeks of weaning. This might have been caused by the lower starter intake during the week before weaning was initiated (0.22 vs. 1.38% BW), which in turn could be related to the higher amount of milk replacer provided in our study compared to what was offered by Eckert et al. (2015). However, the present study brings more evidence that with the provision of proper management and nutrient supply, the extent to which these postweaning ADG are reduced can be minimized. Additionally, despite the reduced gain during weaning when compared to the preweaning period, ADG in this study surpassed or equalled the rates of gain reported for calves under restricted feeding, even during the postweaning period (Kertz et al., 1979, Khan et al., 2007). Thus, the relative reduction of ADG during weaning should not discourage dairy farmers to provide more nutrients to their calves from milk or milk replacer prior to weaning.

Feed efficiency decreased as calves progressed in the feeding program, from  $0.72 \pm 0.01$  preweaning, to  $0.52 \pm 0.01$  during weaning, and finally  $0.44 \pm 0.01$  postweaning. These parameters were unaffected by treatment ( $P > 0.45$ , Table 2). The preweaning feed efficiencies are similar other studies where calves followed an enhanced feeding program based on a high protein and low fat milk replacer (Diaz et al., 2001, Bartlett et al., 2006). Feed efficiencies obtained during the weaning and postweaning periods in this study are significantly higher than reported in other studies (Terré et al., 2006a, Khan et al., 2007, Stamey et al., 2012, Eckert et al., 2015). These differences could be due to the more gradual weaning protocol and the nutrient balance and palatability in the formulated starter. The effect of balancing for all essential amino acids in supporting the observed intake and feed efficiency is intriguing and further work is needed to explore what allowed for the greater efficiencies. Stature measurements (Figure 1C) and rates of gain (data not shown) did not differ among treatments ( $P > 0.20$ ). Overall rates of gain were  $0.23 \pm 0.02$  cm/d for withers and  $0.25 \pm 0.02$  cm/d for hip height.

#### Hematocrit, Plasma BHB and PUN

The hematocrit, plasma BHB and PUN changed with age ( $P < 0.001$ ) but were not affected by treatment ( $P \geq 0.59$ ). Circulating BHB (Figure 1D) and starter intake were well correlated ( $r = 0.82$ ;  $P < 0.001$ ) during the treatment period, supporting the idea that starter was being fermented and consequently butyrate was being produced ruminally. This association and the fact that starter intake was similar among treatments, explains the lack of effect of vitamin supplementation on BHB concentration. However, at the same level of intake of dry feed, calves in our study seem to have higher circulating BHB than

reported in other experiments, where BHB levels range from 0 to 0.4 mmol/L (Eckert et al., 2015, Deelen et al., 2016). Differences in carbohydrate content and fermentability of the solid feed might account for this discrepancy, however, limited feed chemistry is reported in these studies. The increasing levels of BHB and folates observed in plasma confirm that functional development of the rumen started before weaning was finished.

Dumoulin et al. (1991) concluded that during the weeks preceding and following weaning, folates supplied by the diet and ruminal synthesis were not optimum for dairy heifers. They observed improvements in solid feed intake, hematocrit levels and growth post-weaning in calves supplemented with folic acid. When folic acid was added to the milk replacer of rapidly growing white veal calves, growth and hematocrit were improved while feed intake remained unchanged (Lévesque et al., 1993). Further, in dairy heifers, the supplementation of vitamin B<sub>12</sub> during the rearing period did not positively impact growth performance of calves (Grace et al., 2014). Studies examining the supplementation of the other B-vitamins and choline in dairy calves are limited. The literature available and used to determine the NRC (2001) B-vitamin and choline recommendations, were concentrated on determining the lowest dietary intake level to prevent deficiency symptoms during the preweaning phase disregarding optimal performance of calves under restricted feeding conditions (Wiese et al., 1946, Johnson et al., 1947).

The B-vitamin and choline requirements depend on the levels of several nutrients in the diet (McDowell, 2000), and the discrepancy in the effect of supplemental vitamins between the previously reviewed studies and ours could be due to the availability of nutrients and the nutrient balance provided. For example, Judson et al. (1982) observed a positive response in growth to vitamin B<sub>12</sub> supplementation in beef calves, but the basal diet appeared to be cobalt deficient. The dairy heifers in the study by Dumoulin et al. (1991) were fed at least half the amount of the liquid feed offered in our study and were weaned 2 wk earlier, which could have led to lower folate status and a higher susceptibility to inadequacy during weaning. Additionally, the CP content of the starter used in the present study was higher than the one used by Dumoulin et al. (1991) which, in addition to the effort to amino acid balance, might have reduced the calf's need for folate, vitamin B<sub>12</sub>, pyridoxine, niacin, riboflavin and choline, related to amino acid transamination, and methionine remethylation and transsulfuration (Girard and Matte, 2005).

## CONCLUSIONS

The quantities of B-vitamins and choline blends supplemented, as non-protected or rumen-protected forms, improved plasma vitamin B<sub>12</sub> status postweaning, but plasma folate status was unchanged. However, the supplements did not affect growth, dry matter intake, feed efficiency or other indicators of adequacy in dairy calves fed an enhanced plane of nutrition. Supply and utilization of these vitamins by the calf seem to be influenced by the change from a liquid to a solid diet and the respective adaptations to the ruminant state. Collectively, our results suggest that, under the conditions of the study, dairy calves could obtain sufficient B-vitamins and choline from the diet and rumen



synthesis to support optimal performance and part of this response might have been due to the AA balance and profile available in the starter.

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# **GENETIC ENGINEERING IN FIELD CROP AND FORAGE PRODUCTION: CROPPING CONSIDERATIONS FOR HERDS CONSIDERING NON-GMO PRODUCTION**

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## **INTRODUCTION**

Over the past two decades a number of genetically engineered (GE) field crops have been released for commercial production, with nearly all being utilized in the dairy industry as sources of forage and concentrate feed. The adaption by crop growers has been rapid as these crops offer a range of management benefits and conveniences for common challenges troubling the production of key field crops.

While the public dialogue has centered on the term genetically modified organism (GMO), this term characterizes a much wider spectrum of naturally occurring and man made changes to living organisms well beyond the scope of GE techniques. In this paper the terms GE and transgenic are used as they more accurately describe how the crops traits addressed here were derived.

With the rapid adoption of these technologies has come a level of misunderstanding and mistrust from the general public. A phenomenon that has negatively impacted consumer acceptance of foods derived from production systems utilizing GE crops. This is despite the lack of peer reviewed studies showing any evidence that these technologies expose the food system to any safety concerns and the lack of evidence they have created any negative effects on human or animal health in the over two decades since their introduction (NASEM, 2016).

The dairy industry has faced significant stress in balancing growing supply with less robust demand leaving many farmers with an increasingly volatile and unstable milk market. Additionally, due to these supply and demand issues food companies continue to look for marketing opportunities that differentiate their products. This has included the strategy of labeling the absence of an ingredient or production technique, a strategy that often misleads consumers into believing the presence of that particular ingredient or technique is somehow unhealthy or dangerous. This practice adds to the misconceptions surrounding food production and threatens the availability of safe and accepted production technology and techniques.

GE crops have become a target of this labeling practice, with the Danone Food companies "The Dannon Pledge" (Dannon, 2017) being one of the most significant. This leaves some farmers with the tough decision of forfeiting a valuable management tool in an attempt to insulate their business from some of the volatility in commodity markets. Additionally, farms may be able to realize a price premium for agreeing to production

practices excluding the technology, a point that should not be lost as forfeiting an important management tool provides significant justification for additional compensation.

For dairy products it should be noted that milk is a natural product with no genetic modification. Furthermore, the animal products (milk and meat) produced from cows fed GE ingredients are not altered in any way compared to these same products from cows fed a diet containing no GE ingredients.

Unfortunately, as has been the case with other marketing strategies which exclude modern production techniques, what starts as an opportunity for a small percentage of milk producers can have far greater impacts on the industry. Often absence labeling results in many misconceptions about food safety and an erosion of consumer confidence in the approved technologies. The risk exists that over time all producers could lose access to the technology, and the associated premiums for forfeiting the technology, as market share for products produced with the technology diminish.

## HISTORY AND TRAIT OPTIONS

After several years in development the first GE field crops were approved for use in the United States (U.S.) in 1995 with commercial acreage planted in 1996. Since that time the number of different crops as well as the number of pest protection traits available has increased. The list of field crops with an herbicide and or insect pest tolerance trait include corn, soybeans, cotton, canola, sugar beets and alfalfa.

The first herbicide tolerance genes introduced into a field crop were designed to allow the crop to tolerate an application of the broad spectrum herbicide glyphosate, under the Roundup Ready trademark. Currently, tolerance traits for glyphosate, glufosinate, dicamba and 2,4-D are commercially available in certain field crops.

Insect resistance traits are derived from the bacterium, *Bacillus thuringiensis* (Bt). A gene from the Bt bacterium is incorporated into a crop and produces an insecticidal crystalline (Cry) protein that is toxic to certain groups of insects when ingested. It is important to recognize that each Cry protein's effect is specific to a certain group of insects and will not affect other insects or outside of the targeted group or mammals. Numerous Cry proteins have been identified with activity against the larvae of certain types of moths, flies and beetles (Hardee, 2001).

Drought tolerance traits have also been introduced into field crops; however, not all drought tolerance traits are derived from GE techniques with some coming out of conventional plant breeding techniques.

Currently it is common to find seed options where multiple traits are stacked in a single crop variety or hybrid. A common example of this is a triple stacked corn hybrid, a corn hybrid containing a gene for glyphosate tolerance as well as having two different Bt proteins, one for European Corn Borer and one for Corn Rootworm.

The approval and introduction of Roundup Ready (herbicide tolerant) alfalfa in 2006 was an early example of the expansion of this technology from annual crops to a perennial crop. While alfalfa remains the only example in field crops there are other perennial GE crops used in the turf industry. The release of low lignin alfalfa in 2016 marked a shift from GE use as a pest management tool in field crops to a role in increasing the feed quality of a forage specific crop.

## A MANAGEMENT TOOL

The introduction of GE crops added a valuable new option to the crop management toolbox and offers a number of potential benefits in management flexibility, cost, producer safety and environmental impact. However, this does not mean that GE crops are the only management options or even the most economical for a specific situation. It is quite easy to generate real world field scenarios related to pest pressure and growing conditions where GE crops have a large benefit on field performance, while in other common real world scenarios their use may not be warranted or provide any clear benefits to the field's outcome. Fernandez-Cornejo et al. (2006) report increased returns from the adoption of Bt corn and cotton when pest pressure is high, highlighting the effectiveness of these management tools against their targeted pest.

Cultural and chemical control options, with varying levels of effectiveness, existed prior to the availability of GE crops and continue to be viable options for management. Additionally, new cultural, biological and chemical control options have continued to be developed since the advent of GE technology and the need to continue their development in parallel with GE technology remains very important for the long term sustainability of all crop management tools. A report from the National Academies of Science, Engineering and Medicine (NASEM) states, "Genetic engineering and conventional breeding are complementary approaches, and more progress in crop improvement will be made by using both conventional breeding and genetic engineering than by using either alone.", (NASEM, 2016).

The key to the sustained efficacy and availability of each of these management tools is having continued access to all of them. Access to all available tools and utilizing each tool where it presents the best fit for managing a field will reduce the chances of pest developing resistance to any one tool and is consistent with the principals outlined in Integrated Pest Management (IPM). The New York State IPM website states, "Integrated pest management rarely relies on just one tactic—it integrates tactics to prevent pests entirely or reduce them to levels you can live with.", (NYSIPM, 2017).

## UTILIZATION BY FARMS

GE crops dominate U.S. acreage of field corn, soybeans and cotton (Figure 1) with GE Canola and Sugar Beets also representing significant acreage. In 2017, U.S. acreage of GE field corn, soybeans and cotton all exceeded eighty percent with

herbicide tolerant soybeans leading the way, representing ninety four percent of U.S. soybean acres (USDA-ERS, 2017).

Additionally GE alfalfa acreage are expected to grow with the introduction of the Low Lignin trait which improves the fiber digestibility of the plant. Currently the Low Lignin trait is only available stacked with the herbicide tolerance (Roundup Ready) trait and is marketed under the tradename HarvXtra.

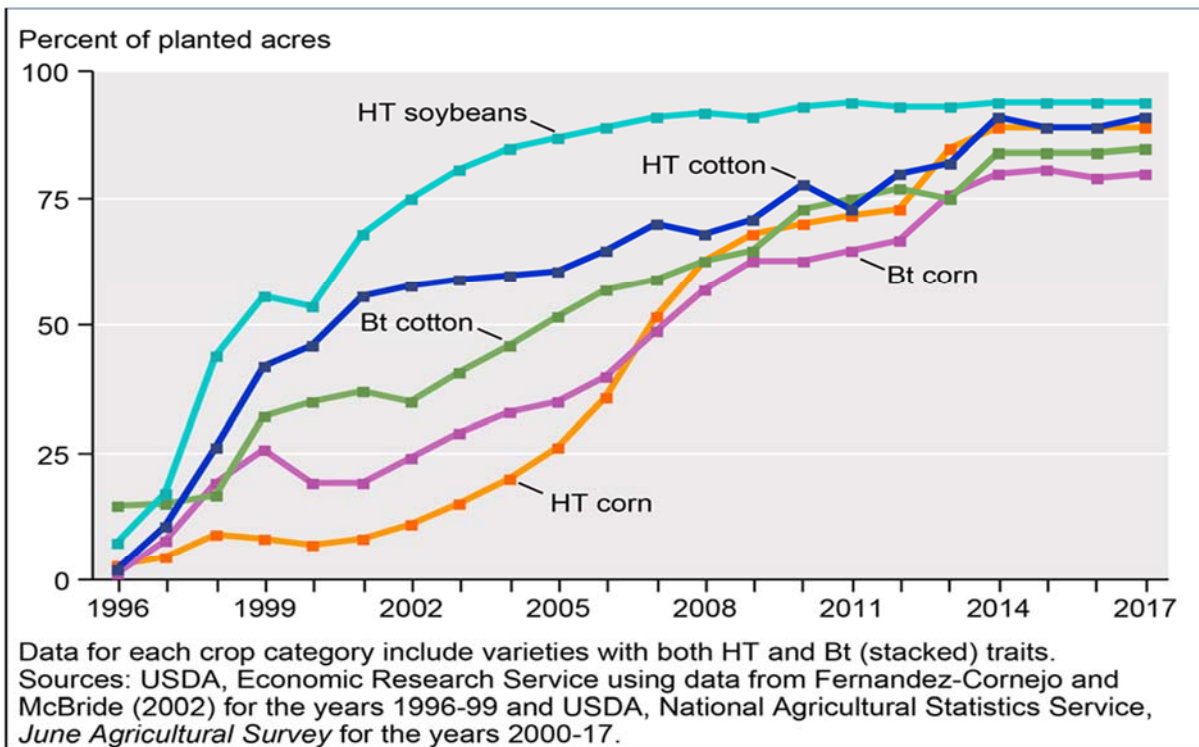


Figure 1. Adoption of genetically engineered crops in the United States, 1996-2017. Source: Recent Trends in GE Adoption, USDA-ERS

While the infinite range of field specific outcomes has made it difficult to document the overall impact of GE technology on the yield (NASEM, 2016; Zulauf and Hertzog, 2011a) and economics of crop production, the overall positive impact of GE crops is generally accepted. “At the farm level, soybean, cotton, and maize with GE herbicide-resistant or insect-resistant traits (or both) have generally had favorable economic outcomes for producers who have adopted these crops, but there is high heterogeneity in outcomes.”, (NASEM,2016). A meta-analysis by Klumper and Qaim (2014) reported that on average farmer adoption of GE crops reduced pesticide use while increasing yields and profits, with greater gains realized in developing countries.

In addition, GE crops can play an important role in farmer adoption of other practices recognized as critical to agricultural sustainability. The use of reduced or no-tillage and cover crops are well established as important practices in long term agricultural and environmental sustainability (Gould et al., 1989; Hobbs et al., 2008). However, these practices also present additional challenges associated with pest

management such as increased plant residue which attracts certain insect pest and the termination of living cover crops required when establishing the subsequent field crop.

As with the pest management options discussed above it should be noted that these conservation practices were implemented prior to the availability of GE crops and remain viable practices without GE technology. However, the availability of GE crops as a management tool greatly expands the options for control and flexibility in control timing that can be critical to successfully managing these practices with the wide range of weather events and growing conditions present in any given season.

### Pesticide Usage Trends

With the widespread adoption of GE crops questions have arisen about the potential overuse of these technologies and corresponding management practices. One aspect of this debate centers on the total pounds of pesticides used annually. GE crops have been touted for their role in reducing pesticide use; however, this has not necessarily been the case as it is necessary to dig beyond the total pounds of pesticides used to understand the changes associated with GE crops.

In a study of total pounds of pesticide active ingredients used in 21 major crops in the U.S. from 1960 to 2008 Fernandez-Cornejo et al. (2014) found that by 2008 total usage, in pounds of active ingredient, had trended downward since its peak in 1981 but had year to year fluctuation. The authors credit this downward trend to a number of factors including the adoption of GE crops. Klumper and Qaim (2014) noted that “Yield gains and pesticide reductions are larger for insect-resistant crops than for herbicide-tolerant crops.”

In a study of U.S. corn and soybean producers from 1998 to 2011 Perry et al., (2016) found that the total amount of soybean herbicides increased by 28% compared to farmers who had not adapted GE crops, while when comparing adopters of GE corn to non-adopters, herbicide use dropped by 1.2% and insecticide use dropped by 11.2%. A second step taken by Perry et al. (2016) was to adjust these pesticide numbers based on the environmental impact quotient (EIQ) of each pesticide. When adjusted for the EIQ the study found that soybean herbicide use by adopters of GE crops was equal to non-adopters while corn herbicide use dropped by 9.8% and corn insecticide use decreased by 10.4%. This highlights a key argument associated with GE crops which suggest that while total pesticide use is not necessarily down in every category, in certain crops the environmental impact of the pesticides being used has lessened.

### Pest Resistance

Aside from general usage trends a consequence of the increased use of GE crops has been an increased reliance on certain pesticide active ingredients that are compatible with the GE crops. This has led to documented pest resistance to both the GE trait as is the case with populations of western corn rootworm that are resistant to



the Cry3Bb1 Bt protein (Gassmann et al., 2011) and an increase in the number of herbicide resistant weed populations (Heap, 2016).

The convenience of use created by GE crops in conjunction with the economic pressures on grain and livestock farmers have had an impact on the development of resistant pest populations. In years of very low margins growers often look for the least cost option and when put under financial stress for consecutive years these least cost options may be used repeatedly despite the known risk that repeated use could increase the chances of pest developing resistance. This is notable in the use of glyphosate for broad spectrum weed control in corn and soybean rotations where both crops are glyphosate tolerant.

In the case of corn rootworm resistance to corn with the Bt trait, a combination of factors related to crop management logistics, financial considerations, inadequate crop rotations and improper implementation of Bt refuge areas in the field has contributed to the current resistance issues.

The industry has worked to address this by developing seed trait packages that include multiple modes of action (MOA) for the control of a target pest as well as premixed pesticide packages also offering multiple MOA for application on targeted pest populations. While this does offer farmers a means of combating resistance development, its effectiveness in combating resistance is limited. Having multiple MOA present is only useful if all MOA are still effective against the pest. Pairing an effective MOA with one that the target pest population is already resistant to only places further selection pressure on the MOA that is working (Shields, 2017). These technological solutions need to be paired with greater use of other currently available management tools to enhance their effectiveness.

It is difficult to pin the blame for resistance issues in any one place; however, it is clear that they are becoming an increasing threat to the viability of GE crops and need to be addressed by the industry to assure the continued availability of GE technology as a management tool in an integrated approach to crop management. Without a greater emphasis on the stewardship of these technologies the discussion regarding their voluntary forfeiture for potential marketing advantages becomes a moot point.

## PREVALENCE OF GE CROPS IN DAIRY RATIONS

In addition to the substantial acreage of GE corn grown for on-farm use as silage and grain and the growing interest in GE alfalfa, the high percentage of acres growing major feed commodities such as corn, soybeans, cotton and canola at the national level assures that feed ingredients derived from GE crops are common on any dairy farm that is not actively choosing to exclude them.

Consequently a dairy farms transition away from GE crops and feed ingredients will include changes to the entire supply chain of major feed ingredients well beyond any home grown forage or grain crops (Chase, 2017).

A list of field crops relevant to dairy production and the corresponding traits that are derived from conventional and GE techniques was developed for a PRO-DAIRY Forage Management sheet (Lawrence, 2017) and is presented here (Figure 2).

Genetically Engineered	Conventional
<b>CORN</b>	
<ul style="list-style-type: none"> <li>• Herbicide Tolerance               <ul style="list-style-type: none"> <li>○ Glyphosate tolerance                   <ul style="list-style-type: none"> <li>▪ Roundup Ready (RR)</li> <li>▪ Glyphosate Tolerant (GT)</li> </ul> </li> <li>○ Glufosinate tolerance                   <ul style="list-style-type: none"> <li>▪ Liberty Link (LL)</li> </ul> </li> <li>○ 2,4-D tolerance                   <ul style="list-style-type: none"> <li>▪ Enlist</li> </ul> </li> <li>○ Dicamba tolerance                   <ul style="list-style-type: none"> <li>▪ Roundup Ready Plus Extend</li> </ul> </li> </ul> </li> <li>• Bt Insect Protection               <ul style="list-style-type: none"> <li>○ Corn Rootworm</li> <li>○ Lepidoptera (Moths &amp; Butterflies)</li> </ul> </li> <li>• Drought Tolerance               <ul style="list-style-type: none"> <li>○ <i>SOMETIMES</i>, check with seed supplier</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• Brown Mid Rib (BMR)</li> <li>• Floury Starch Silage Hybrids</li> <li>• Disease Tolerance</li> <li>• Drought Tolerance               <ul style="list-style-type: none"> <li>○ <i>SOMETIMES</i>, check with seed supplier</li> </ul> </li> </ul>
<b>SOYBEANS</b>	
<ul style="list-style-type: none"> <li>• Herbicide Tolerance               <ul style="list-style-type: none"> <li>○ Glyphosate tolerance                   <ul style="list-style-type: none"> <li>▪ Roundup Ready (RR or RR2)</li> <li>▪ Glyphosate Tolerant (GT)</li> </ul> </li> <li>○ Glufosinate tolerance                   <ul style="list-style-type: none"> <li>▪ Liberty Link (LL)</li> </ul> </li> <li>○ 2,4-D tolerance                   <ul style="list-style-type: none"> <li>▪ Enlist</li> </ul> </li> <li>○ Dicamba tolerance                   <ul style="list-style-type: none"> <li>▪ Roundup Ready Plus Extend</li> </ul> </li> </ul> </li> <li>• High Oleic               <ul style="list-style-type: none"> <li>○ <i>SOMETIMES</i>, check with seed supplier</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• Disease Tolerance</li> </ul>
<b>ALFALFA</b>	
<ul style="list-style-type: none"> <li>• Herbicide Tolerance               <ul style="list-style-type: none"> <li>○ Glyphosate tolerance                   <ul style="list-style-type: none"> <li>▪ Roundup Ready (RR)</li> </ul> </li> </ul> </li> <li>• Low Lignin               <ul style="list-style-type: none"> <li>○ HarvXtra</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• High Quality (HQ)</li> <li>• Low Lignin (other than HarvXtra)</li> <li>• Hybrid</li> <li>• Multifoliate</li> <li>• Potato Leafhopper Tolerance</li> <li>• Alfalfa Snout Beetle Tolerance</li> <li>• Disease Tolerance</li> <li>• Branch Rooted</li> </ul>
<b>COTTON</b>	
<ul style="list-style-type: none"> <li>• Herbicide Tolerance</li> <li>• Bt Insect Protection</li> </ul>	
<b>CANOLA</b>	
<ul style="list-style-type: none"> <li>• Herbicide Tolerance</li> </ul>	
<b>SUGAR BEETS</b>	
<ul style="list-style-type: none"> <li>• Herbicide Tolerance</li> </ul>	

Figure 2: Crops Relevant to Row Crop, Dairy and Livestock Production

## MANAGING CROPS WITHOUT GENETIC ENGINEERING TECHNOLOGY

As discussed, in the majority of situations viable management alternatives to GE technology exist and their use in conjunction with biotech is encouraged in an integrated management approach. Additionally a number of important crop traits, ranging from disease resistance to improved quality, continue to be available through conventional plant breeding (Figure 2). With that said, the absence of these management tools creates a void in the crop management toolbox that may expose a crop production program to increased variability in yields, cost and environmental impact.

While a direct increase in yield from the adoption of GE crops is hard to quantify and Zulauf and Hertzog (2011b) found that the decline in yield variability was not unique to GE crops when compared to non-GE crops in their study, The National Academy of Science, Engineering and Mathematics did note that GE crops have facilitated a narrowing of the gap between a crops yield potential and actual yield (NASEM, 2016).

Given the desirable characteristics of GE crops in addressing defined management challenges related to growing conditions, their use is likely to increase in importance in stabilizing production levels and lessening the chances of major crop failures as farmers have experienced an increased frequency in extreme weather events during critical periods in a cropping season. Strategic planning will be needed to compensate for the absence of these tools and minimize the potential upturn in production variability. For individual farms, considering a shift to a non-GE production system, it will be critical to carefully evaluate the expected economic impacts specific to their operation and what level of price premium is necessary to justify this shift.

### Weed Management

In the absence of herbicide tolerate crops growers will need to rely heavily on a well-planned pre-emergence (PRE) herbicide program to minimize weed impact. This will necessitate a greater understanding of specific weed populations in each field to customize the PRE program to the weeds present. While post emergence (POST) products exist for control of emerged weeds in non-herbicide tolerant crops their cost, specificity and window of effectiveness are all highly variable relative to the options present in herbicide tolerant crops.

The herbicide options present for non-herbicide tolerant corn often have a longer residual time in the soil and may also carry additional crop rotation restrictions that will need to be accounted for in crop management plans.

### Insect Management

The control of key insect pest in the absence of Bt crops will require an emphasis on short crop rotations and an increased reliance on insecticides. Corn Rootworm, a major pest in almost all corn producing areas of the U.S. (Marra, 2012), can be effectively controlled using crop rotation, though the practicality of this does not always

fit into overall farm management strategies and can be limited by available crop acreage, geography and rotation resistant variants of the insect. Western Bean Cutworm, a relatively new pest of concern in corn production is not affected by crop rotation leaving treatment with insecticide as the leading alternative for control.

Increased insecticide usage in the form of planter application may necessitate additional application equipment on planters. Post emergence control measures may require additional passes over the field which can cause additional physical damage to the crop and may require specialized high clearance spray equipment. Farm owners and employees may need to hold valid pesticide application licenses in their state to legally apply insecticides required in the absence of Bt crop traits.

### Drought Management

The availability of drought tolerance traits are relatively new in the history of GE crops but present an important tool in reducing the impact of increasingly variable weather patterns. The use of GE methods to develop these drought tolerance traits are company specific. If a grower desires drought tolerant traits it will be important to verify with each company the method in which the trait was added to the plant and if it qualifies as not being derived from genetic engineering.

## FUTURE USES OF GENETIC ENGINEERING

In any discussion regarding the implications of product marketing and consumer perception on a producer's access to and use of a technology it is critical to not only consider the current applications of said technology but also the future potential. GE techniques continue to be employed in a wide array of uses, not only in field crops but also in fruit and vegetable crops central to human nutrition. Additionally the applications of the technology continue to reach beyond their common use in field crop pest management to altering the nutritional profile of plants, both for direct human consumption and in forages for livestock. Moving away from GE management tools should be looked at in the context of not just the current technology that will be forfeited but also how it will jeopardize the development and accessibility of future crop enhancements.

### Forage Quality

Improving forage quality remains a key focus in dairy production and continues to be addressed at the field management and plant breeding level. An example of this was noted in a 2016 webinar presented by Hay and Forage Grower Mark McClaslin of Forage Genetics International was asked about future developments in improving the forage quality of alfalfa. He responded that one of the most promising advancement currently being worked on is the introduction of tannins into the alfalfa plant that would slow the rate of protein degradation in the rumen, thereby increasing the bypass protein

available from the plant allowing ruminant animals to utilize a greater percentage of the protein available in the alfalfa plant (McClaslin, 2016).

## Gene Editing

The gene editing technology commonly referred to as CRISPR, an acronym for clustered regularly interspaced short palindromic repeats, is garnering great interest in applications ranging from reducing disease susceptibility in plants to addressing genetic diseases in mammals, including humans. This technology is generally considered to hold great potential in a range of applications (Folta, 2016; Zaidi, 2017; Zhang, 2016).

From a scientific standpoint the precise method in which CRISPR edits a targeted part of the genome is very different than GE (Folta, 2016); however, it remains unclear how the general public will differentiate the two techniques and what their acceptance of gene editing will be in comparison with GE.

## CONCLUSIONS

The ability of farmers to produce crops for GE free markets is feasible with the economic and environmental outcomes of doing so highly tied to economic premiums for the product and specific growing conditions, which will vary by farm and growing season. Producers considering this production system should recognize the added planning and management required, the chances of increased production and cost variability and the broader implications these decision may have on the access to these technologies in the future of food production.

Currently available genetic engineering techniques and emerging techniques in gene editing offer producer and consumers a range of potential benefits in the efforts to increase the sustainability of food production. Responsible management of these technologies requires; continual advancement of technologies, sound and on-going scientific review of their safety and effectiveness, producer accountability in proper use of technologies, public confidence in the scientific process, food chain support of sound production practices. Additional in-depth analysis to better understand the economic impacts of GE technology both at the farm level and the global societal scale is needed.

It is critical the entire supply chain from crop developers and growers to food manufacturers and consumers be engaged in a scientifically based dialogue regarding the role of this technology in food production and the implications of using exclusionary tactics in marketing food.

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## CONSIDERATIONS FOR DEVELOPING NON-GMO DAIRY RATIONS

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### INTRODUCTION

A number of dairy food processing companies are developing and marketing non-GMO products. Thus, they are enlisting dairy herds to adjust their feeding programs to use non-GMO forages and feeds. This shift in providing non-GMO dairy products is based on meeting demands of consumers. Many of these dairy food processing companies acknowledge that there are no differences in the safety of foods produced using GMO technology. A review paper evaluated the impact of GE (genetically engineered crops fed to animals (Van Eenennaam and Young, 2014). They reported finding no trends in livestock health and productivity that could be related to feeding GE derived crops. This same study also indicated that there were no differences in the nutritional profiles of products from animals fed GE rations. A publication from the National Academy of Science concluded that there was no substantial scientific evidence that foods from GE crops were any less safe when compared to foods from non-GE crops (NAS, 2017).

### MILK PRODUCTION

Ferraretto and Shaver (2015) used a meta-analysis approach to examine potential differences between GMO hybrids and their non-GMO isogenic hybrids. This study included 48 research papers and 162 treatment means. There were 21 treatment means for the isogenic hybrids and 13 treatment means for GMO hybrids. The authors concluded that forage nutrient composition and milk production was similar for both types of hybrids.

### FEEDS AVAILABLE

The primary GMO crops grown in the U.S are corn, alfalfa, cotton, soybeans canola and sugar beets. A companion paper in these proceedings provides detail on the percent of the crop acres planted to GMO crops (Lawrence 2017). This same paper also identifies forage sources as either GMO or non-GMO (Lawrence, 2017).

However, there are also non-GMO seeds available for most of these crops. The following is a short list of non-GMO feeds available for use for use in dairy rations (Non-GMO Sourcebook, 2017). These feeds may be have limited availability.

- Corn grain, hominy feed, distiller's grain, corn gluten feed, corn gluten meal.
- Soybean meal, expeller soybean meal, soy hulls, roasted soybeans.
- Beet pulp.
- Canola meal, expeller canola meal.
- Alfalfa pellets.



Wheat, oats and barley should also qualify as non-GMO since no GMO varieties are grown in the U.S. The milling products from these grains should also be non-GMO.

## RATION COMPOSITION

Rations for animals fed non-GMO feeds will be formulated using your current software and nutrient composition guidelines. The primary difference is that each forage and feed needs to be identified as either GMO or non-GMO. The percent of the total ration dry matter needs to be determined for each feed. Any feed added to the ration that comprises <5% of the total ration dry matter does not need to be non-GMO and is not evaluated in the auditing process (Heyman, 2017; Non-GMO Project, 2017). The inclusion level for these feeds must be verified both by formulation and actual batch feed mixing records. Ingredients such as animal protein sources, added fats and amino acids can be used as long as each is <5% of the total ration dry matter. Multiple feeds can be used if each added feed is <5% of the total ration dry matter. As an example, a ration could contain 4% soy hulls (GMO), 3% animal protein blend, 4% expeller soybean meal (GMO), 1.7% minerals and vitamins, 0.9% bypass fat and 0.1% amino acids. Total ration cost will increase since non-GMO feeds are priced higher than the same feed from a GMO source. These guidelines apply to rations fed to replacement heifers, dry cows and milking cows. These rations need to be fed for a minimum of 30 days before non-GMO certification could be obtained. Any ingredient that comprises >5% of the total ration dry matter must be non-GMO verified or tested.

## RECORD KEEPING

One of the biggest changes in management of non-GMO rations is record keeping requirements. The actual records needed may vary some depending on the milk processor purchasing the milk. There may also be more specific guidelines provided by the third part auditing firms involved in the process.

1. Forage and feed purchase records. Information needed includes supplier, date of purchase, quantity purchased and storage location. This entails saving all invoices, receipts, feed tags, delivery weight slips or information on forage seed bags.
2. Verification – Letters are required from suppliers if they are providing a non-GMO certified product.
3. Forages – Track the hybrids or varieties purchased, field maps as to where the seed was planted, planting date, harvest date quantity harvested and storage location. Aerial maps of forage storage structures may help or be required.
4. Feed ingredients – Supplier purchased from, date of purchase, tons and storage location. The GMO or non-GMO status of each feed needs to be recorded.
5. Rations – All rations for replacement heifers, dry cows and milking cows must be included. The quantity of each feed in the ration and percent of the ration dry matter supplied by each ingredient. A record of the GMO or non-GMO status of each feed is needed. Data on animal numbers and milk production are needed.

6. Feeding management – Actual dry matter intakes are needed. This involves tracking the quantity of feed fed and adjusting for refusals. The simplest way to do this is to use one of the electronic feed management systems.
7. Test results – Copies of any tests done on individual feeds to confirm non-GMO status. There are on-farm tests available for corn, cotton, alfalfa and soybeans. A feed sampling and testing protocol needs to be developed.
8. Separation – GMO and non-GMO forages and feeds need to be stored separately to prevent any contamination.
9. Third party verification – Most processors are working with an outside firm to provide on-farm audits. The records listed above should provide the base information needed by the auditors. However, there may be additional records or information required by specific auditing firms.

## SUMMARY

Developing rations for non-GMO herds uses the same principles and procedures that are currently used by nutritionists. There may be some limitations in ingredients available for use in non-GMO rations. Ration cost will usually be higher due the higher cost of non-GMO ingredients. The major change at the farm level is an increase in the type and quantity of the records that need to be kept on forages, feeds, rations formulated and rations fed. This may require development of an on-farm record keeping system to achieve this goal. Total costs for feeds and management time will increase and need to be compensated for by a premium pricing system for milk.

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## FEEDING THE FRESH COW: FIBER CONSIDERATIONS

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### INTRODUCTION

The transition from pregnancy to lactation is a time of great metabolic adaptation for the dairy cow that can often end in less than ideal circumstances. With the onset of lactation, the demands for glucose, amino acids, and fatty acids almost double (Bell, 1995) and coupled with the dynamics of DMI, cows are typically in negative nutrient balance just after parturition. In this negative nutrient state body reserves are mobilized as circulating NEFA and BHBA, and utilized as fuels in many tissues to meet energy needs. Elevated levels of NEFA and BHBA in the periparturient period increase risk for diseases such as displaced abomasum, ketosis, and metritis (Ospina et al., 2010). Research has shown that feeding higher energy rations in the immediate postpartum period can reduce negative energy balance and reduce circulating NEFA and BHBA (Rabelo et al., 2003, McCarthy et al., 2015a, McCarthy et al., 2015b). Generally higher energy fresh cow rations contain high amounts of NFC; however, large changes in diet composition, especially in the transition period, can lead to subacute ruminal acidosis (Nocek, 1997, Penner et al., 2007, Williams et al., 2015) and detrimental effects on metabolism and production (Stone, 2004).

The potential to further refine carbohydrate nutrition of fresh cow rations through the use of different pools of fiber has remained largely unexplored. Physically effective fiber has previously been investigated in regards to mitigating SARA through increased rumination and chewing activity (Stone, 2004), though evidence in the immediate postpartum period is lacking. In mid-lactation rations uNDF<sub>240</sub> has recently been investigated as a possible regulator of both dry matter intake and rumen health (Cotanch et al., 2014). In a ration with low uNDF<sub>240</sub> rumen health could be compromised, though with high uNDF<sub>240</sub>, intake can be limited due to gut fill. Previous case study data from the Overton lab would indicate that cows fed higher uNDF<sub>240</sub> (10.7% of ration DM, intake 0.36% of BW) had higher DMI and improved health status when compared to cows fed low uNDF<sub>240</sub> (8.3% of ration DM, intake 0.27% of BW) in the postpartum period (McCarthy et al., 2014c). To the authors' knowledge, there are few published data investigating the effects of modulating uNDF<sub>240</sub> and overall digestion pools in the periparturient period. Gaining insight into the ideal lower and upper bounds of uNDF<sub>240</sub> and digestibility pools in the fresh period could prove valuable for increasing animal health and productivity as sufficient uNDF<sub>240</sub> might help increase DMI during the immediate postpartum period, but excessive uNDF<sub>240</sub> and slowly digestible NDF might limit DMI during the same period.

Another opportunity to increase DMI and overall energy intake during the immediate postpartum period could be to focus on the digestible fiber fraction through use of corn silage hybrids with high NDF digestibility such as brown mid-rib (BMR) corn

silage. Feeding BMR corn silage in the prepartum and postpartum period has been found to increase intake and milk production in the early lactation period (Stone et al., 2012), while feeding BMR only the postpartum period decreased BW loss and increased post-peak lactation milk yield (Holt et al., 2013). The positive milk production effects shown by Stone et al. (2012) were shown to carry over through post-peak lactation even after cows were switched to a conventional corn silage, indicating lasting effects due to changes in intake and energy balance early in lactation. Utilizing all of the different aspects of fiber within postpartum rations could provide a more energy dense ration while also being mindful to rumen health.

#### EXPERIMENT 1: THE EFFECTS OF VARYING uNDF<sub>240</sub> AND peNDF CONTENT OF FRESH RATIONS ON PERFORMANCE AND METABOLISM

Fifty-six multiparous Holstein cows were fed a common prepartum ration beginning 28 d prior to expected parturition. Cows were assigned randomly to one of two postpartum diets differing in content of uNDF<sub>240</sub> and peNDF with randomization restricted to control for parity and previous lactation 305 d mature equivalent milk production. Treatment diets, high fiber (**HF**, n=27) and low fiber (**LF**, n=29), were formulated for equivalent metabolizable protein (MP) and starch, with higher fiber levels achieved through the addition of chopped straw in the HF diet. At 29 days in milk (DIM), HF cows were switched to the LF diet and all cows were fed the LF diet through 42 DIM.

Cows were housed in tie-stalls and fed once daily at approximately 0800 h throughout the study. Individual intakes were calculated by weighing the feed delivered and refused daily. A refusal rate of 10% was targeted daily to allow for ad-libitum intake. All rations were formulated using CNCPS v6.55 and ingredient and analyzed composition of diets are presented in Table 1. Weekly samples of TMR and all feed ingredients were collected for determination of DM which was used to adjust feed inclusion rates and to calculate DMI. At the end of the experiment all retained feed samples were ground and composited by 4-wk intervals over the duration of the study. Composite samples were sent to a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD) for wet chemistry and in-vitro fermentation analysis.

Body weights and BCS were measured weekly throughout the experiment. BCS was assigned by two scorers weekly and averaged for analysis. After calving, all cows were milked 3x daily and individual milk weights were recorded. Once weekly milk samples were collected from three consecutive milkings and sent to a commercial laboratory (DairyOne, Ithaca, NY) for analysis of milk composition and SCC. Milk fat and milk protein were used to calculate FCM and ECM. Weekly energy balance was calculated according to NRC (2001). Rumination time was recorded in 2-h intervals over the duration of the study using rumination collars (HR tags; SCR Dairy, Madison, WI).

Blood samples were collected via coccygeal venipuncture 2x per week prior to parturition, daily from the day of parturition through 7 DIM, 3x per week through 21 DIM and 2x per week through 42 DIM. Plasma was harvested, snap frozen in liquid nitrogen and stored at -20°C until analysis. Samples were analyzed for BHBA, NEFA, and glucose. Liver biopsies were obtained from a subset of 40 cows on d 7 ± 1.1 (mean ± SD) and 14

± 1.0 postpartum and incubated in an *in vitro* system to determine liver capacity to convert [1-<sup>14</sup>C]propionate to glucose and CO<sub>2</sub> and [1-<sup>14</sup>C]palmitic acid to CO<sub>2</sub>, esterified products, and acid soluble products.

Statistical analyses were conducted using the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC). Repeated measures data were analyzed using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996) with model effects of treatment, time, and treatment × time. Covariate measurements collected in the week of enrollment were included in all models.

Table 1. Ingredients and nutrient profile of rations (mean ± SD), obtained through wet chemistry analysis and *in vitro* fermentation.

Item	Diet		
	Prepartum	Low Fiber (LF)	High Fiber (HF)
<b>Ingredients, % of ration DM</b>			
Conventional corn silage	45.21	42.31	38.46
Alfalfa hay	-	10.58	10.58
Straw	20.84	1.15	8.65
Corn meal	2.43	17.64	20.15
Soybean meal	-	6.03	4.73
Wheat middlings	-	4.82	1.58
Amino Plus	5.9	4.34	5.31
Canola meal	3.47	1.61	3.88
Corn gluten feed	1.74	1.61	0.47
Blood meal	2.43	0.95	1.09
Soybean hulls	6.95	2.41	-
Citrus pulp	4.52	-	0.79
Energy Booster	-	1.29	1.58
Rumensin, mg/d <sup>1</sup>	439	365	334
Other	6.4	2.3	2.3
<b>Analyses, % of ration DM</b>			
aNDFom	43.1 ± 0.3	32.8 ± 1.4	35.3 ± 2.3
ADF	29.0 ± 0.5	21.3 ± 1.1	22.9 ± 2.1
Starch	15.6 ± 0.3	24.8 ± 1.7	24.6 ± 2.3
Sugar	3.5 ± 0.4	5.0 ± 0.7	3.9 ± 0.1
Fat	2.3 ± 0.2	3.3 ± 0.2	3.2 ± 0.2
uNDF <sub>240</sub>	12.8 ± 0.5	9.5 ± 0.4	12.2 ± 1.6
peNDF	33.3	21.6	23.2
MP, g/kg DM <sup>1</sup>	89.0	112.1	108.0

<sup>1</sup> Formulated value given by Cornell Net Carbohydrate and Protein System v. 6.55 using actual mean intakes

Intake and production results are presented in Table 2. Postpartum intake was lower for cows fed HF in wk 3 and 4 ( $P < 0.01$ ) compared to cows fed LF. After the diet change, in wk 5 and 6 cows fed HF obtained similar intake as cows fed LF throughout

the experiment. Despite differences in intake, no differences were detected for rumination. Milk yield was lower for cows fed HF in week 4 ( $P<0.01$ ) than cows fed LF, no treatment differences were seen in milk components or ECM.

Table 2. The effect of low fiber and high fiber diets in the early postpartum period on intake, milk yield, and rumination for 1 to 6 wk postpartum.

Item	LF	HF	SEM	P-Value	
				Trt	TrtxTime
Prepartum DMI, kg/d		15.5		-	-
Postpartum DMI, kg/d	23.6	22.2	0.3	0.002	<0.001
uNDF intake, %BW <sup>1</sup>	0.29	0.34	0.01	<0.001	0.047
Milk yield, kg/d	46.2	44.7	1.0	0.26	0.001
Fat, %	3.72	3.87	0.85	0.20	0.14
FCM, kg/d	47.9	47.6	1.1	0.83	0.07
Protein, %	3.09	3.02	0.05	0.30	0.56
Lactose, %	4.78	4.76	0.05	0.66	0.68
Total solids, %	12.57	12.64	0.13	0.70	0.33
ECM, kg/d	48.2	47.3	1.1	0.55	0.12
Rumination, min/d	544	543	8	0.90	0.14

<sup>1</sup>Calculated only for weeks where treatment diets differ (1 to 4 wk)

Blood metabolites and calculated energy balance are shown in Figure 2 (A-D). As expected given intake differences, cows fed HF had had higher NEFA and BHBA, and lower blood glucose and energy balance at different points through the experiment (Trt x Time  $P=0.01$ ). Again, after cows assigned to the HF treatment were fed the LF diet at 29 DIM, cows from both treatment groups had similar blood metabolite concentrations and calculated energy balance by wk 5 and 6.

In-vitro liver incubation results are shown in Figure 3 and Table 3 below. Cows fed HF tended to have higher rates of esterification and lower rates of oxidation of [1-<sup>14</sup>C]palmitic acid, which is consistent with the differences in intake, blood metabolites and overall energy balance discussed above. There were no treatment differences in [1-<sup>14</sup>C]propionate metabolism, however there was an overall effect of day of biopsy for many products which is shown in Table 3. Overall, the conversion of [1-<sup>14</sup>C]palmitic acid to esterified products decreases, CO<sub>2</sub> oxidation increases, and the ratio of Glucose:CO<sub>2</sub> for [1-<sup>14</sup>C]propionate metabolism increases as the cows get later in lactation. This evidence demonstrates the changes in liver metabolism that are occurring as cows are progressing more towards a positive energy balance state.

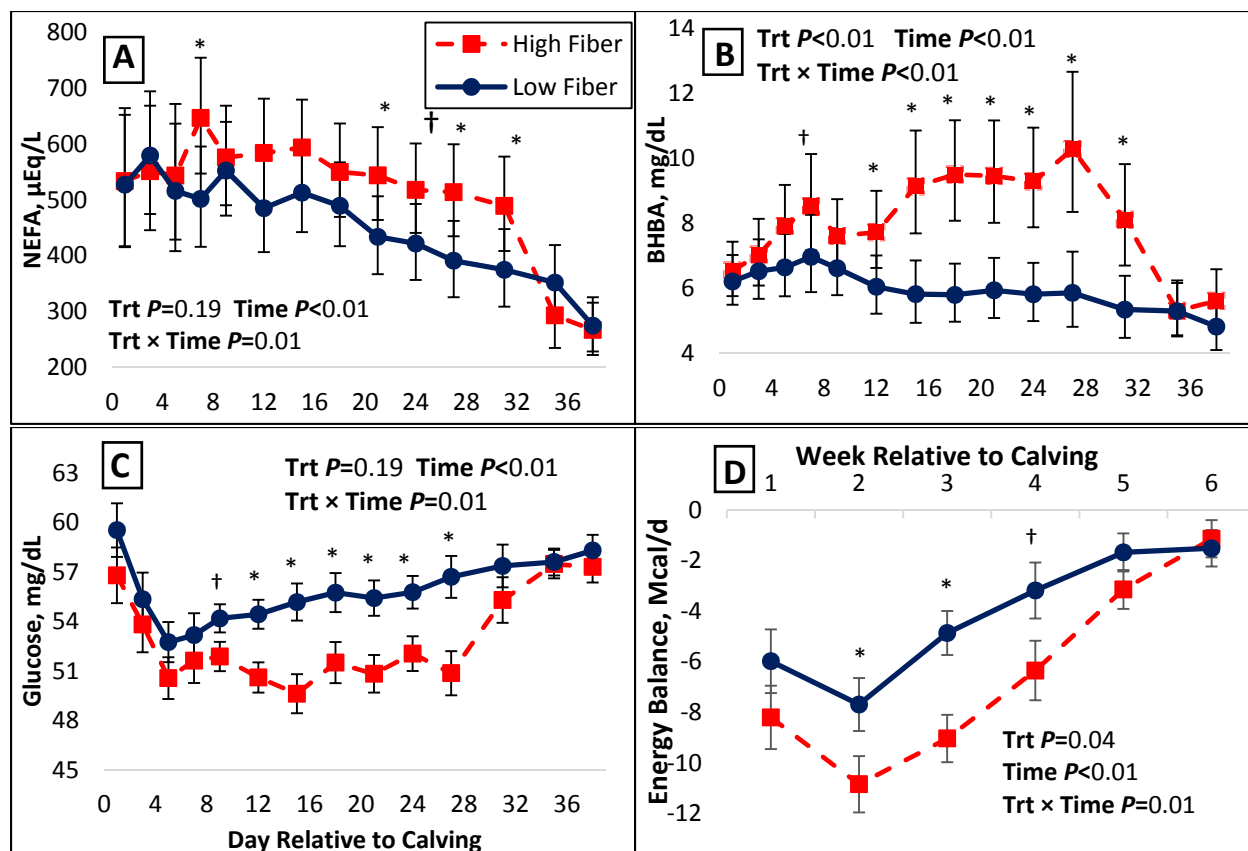


Figure 1. Plasma NEFA (A), BHBA (B), glucose (C), and energy balance (D) by time relative to calving, NEFA and BHBA reported as geometric means with back transformed 95% confidence intervals. Significant differences indicated with an asterisk (\*), trends with a cross ( $\dagger$ ). Energy balance was calculated according to NRC (2001).

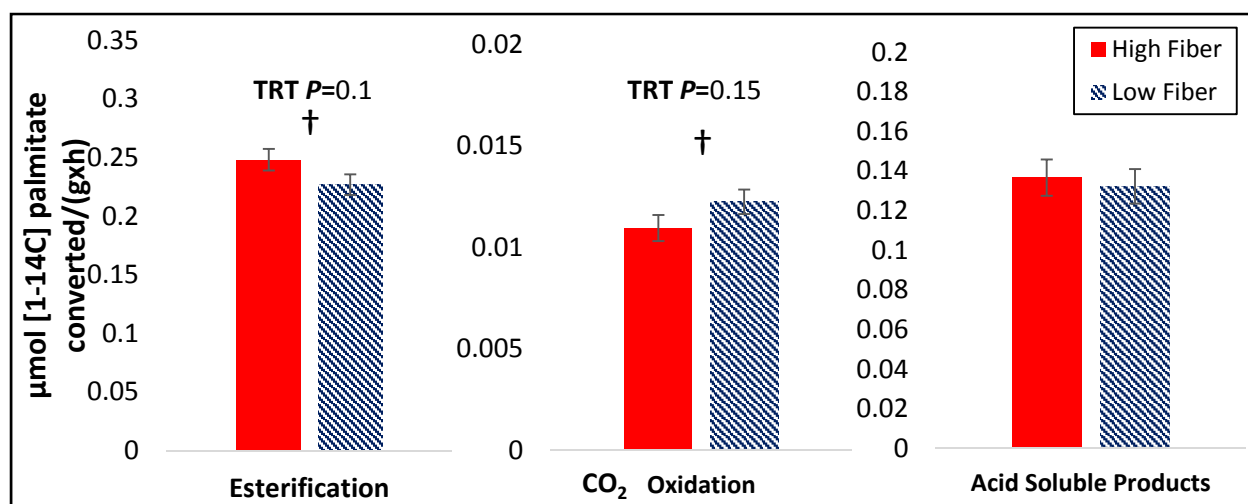


Figure 3. Effect of treatment on rates of conversion of [1-14C] palmitic acid to end products; esterified products, CO<sub>2</sub>, and acid soluble products by bovine liver slices after in vitro incubation. Trends for differences ( $P<0.15$ ) indicated with a cross ( $\dagger$ ).

Table 3. Effect of DIM on mean rates of [1-<sup>14</sup>C]palmitate and [1-<sup>14</sup>C]propionate metabolism by day of biopsy.

Item	Day 7	Day 14	SEM	P-Value	
				Time	Trt×Time
<u>Palmitate</u>					
	-μmol/(g x h)-				
Esterified Products	0.244	0.232	<0.01	0.007	0.364
CO <sub>2</sub> Oxidation	0.010	0.013	0.01	0.076	0.343
Acid Soluble Products	0.133	0.136	0.01	0.681	0.890
<u>Propionate</u>					
Glucose:CO <sub>2</sub>	0.530	0.564	0.02	0.148	0.937

In this trial, the formulated diets focused on uNDF<sub>240</sub> demonstrated that the levels achieved in the low fiber diet (9.5% of DM, 0.29% of BW) were adequate whereas the levels in the high fiber diet (12.2% of DM, 0.34% of BW) were gut fill limiting. It is important to note that, as a result of variation in forage composition, actual uNDF<sub>240</sub> levels were more than one percentage unit higher than intended, and the LF diet in the current study was intermediate to the levels characterized in the McCarthy et al. (2014) study. Though not a definitive limit, these data would suggest a maximum for uNDF<sub>240</sub> intake of ~0.34% of BW in the postpartum period, with an optimum range of uNDF<sub>240</sub> intake ranging from 0.29 to 0.35% of BW or as a percentage of the diet 9.5% to 11% DM. However, it is important that all fiber pools be appropriately characterized to ensure all of the NDF pools are accounted for to fully understand what was first limiting for rumen fill. Overall, cows fed increased levels of uNDF<sub>240</sub> and peNDF resulted in cows that were restricted in intake thus in a more severe negative energy balance. This negative energy balance was reflected not only by blood metabolites, but through impaired liver metabolism as well. However, the detrimental effects of this restriction did not appear to carryover once cows switched to a lower fiber diet. It appears that uNDF<sub>240</sub> and overall forage digestibility and pool size likely plays an important role in regulating intake in the early fresh period, this area warrants further investigation.

#### EXPERIMENT 2: STRATEGIES FOR OPTIMIZING DIETARY ENERGY AVAILABILITY IN FRESH RATIONS

Eighty-five multiparous Holstein cows were enrolled at 28 d prior to expected parturition and assigned to treatments at 21 d before calving in a completely randomized design with a 2 X 2 factorial arrangement of treatments. Cows were randomly assigned to treatment with randomization restricted for parity and previous 305-d mature equivalent milk yield. Treatment variables were corn silage type [conventional corn silage, TMF hybrid (**CON**) vs. brown mid-rib, BM3 hybrid (**BMR**), Mycogen Seeds, Indianapolis, IN] and Rumensin supplementation [0 mg/d prepartum and postpartum (**NO**) vs. 330 mg/d prepartum and 450 mg/d postpartum (**RUM**), Elanco Animal Health, Indianapolis, IN]. For inclusion in the final dataset, cows had to be fed the prepartum treatments for a minimum of 9 d. At parturition, cows were fed a fresh diet formulated to follow their assigned treatment scheme (CON vs. BMR and NO vs. RUM) through 42 DIM. Treatment diets were formulated to be the same except for the type of corn silage or Rumensin mix, ration ingredients and analyzed composition are presented in Table 4. In the dataset 22 cows



were in the conventional corn silage with Rumensin supplementation group (**CON-RUM**), and 21 cows were in each of the conventional corn silage, no Rumensin supplementation (**CON-NO**), BMR corn silage, no Rumensin supplementation (**BMR-NO**), BMR corn silage with Rumensin supplementation (**BMR-RUM**) treatment groups.

In terms of housing, feeding, feed samples, BW, BCS, and milking cows were managed similarly to the previously described experiment with few differences. For feeding, basal TMR was delivered for each period and forage type, containing forages and a base grain. Small inclusion pelleted grain mixes (to deliver RUM or NO) were then added to small batches of the base TMR to be mixed before delivery to the animals. Milk samples were collected 2x weekly for the first two weeks of lactation and weekly thereafter. Milk composition was analyzed in the Barbano Lab at Cornell University using mid-NIR techniques (Barbano et al., 2014), though these data have yet to be analyzed. Blood samples were collected via coccygeal venipuncture 1x per week prior to parturition, 2x per week for the first 2 weeks postpartum, and 1x per week through 42 DIM. Prior to centrifugation and harvesting plasma, whole blood was used to determine BHBA using the NovaVet (Nova Biomedical, Billerica, MA) handheld ketone meter. Plasma was then harvested and stored until study completion for analysis of NEFA.

Prepartum and postpartum data were analyzed and will be presented separately. Statistical analyses were performed using the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC). Repeated measures data were analyzed using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996). Fixed effects were corn type, Rumensin treatment, time, all 2-way interactions, and a 3-way interaction of corn, rumensin, and time. Covariate measurements collected in the week prior to receiving treatment diet were included in all models.

Table 4. Formulated ingredient composition of diets for which ingredients besides corn silage type differed.

Item	Prepartum		Postpartum	
	NO	RUM	NO	RUM
<b>Ingredients, % of ration DM</b>				
Corn silage	51.67	51.67	51.46	51.46
Hay Crop Silage	-	-	10.65	10.65
Straw	23.33	23.33	2.66	2.66
Corn meal	-	-	15.08	15.08
Canola meal	4.57	4.57	6.45	6.45
Amino Plus	4.0	4.0	5.32	5.32
Blood meal	1.67	1.67	1.77	1.77
Wheat middlings	1.93	1.92	1.40	1.39
Soybean meal	0.67	0.67	0.48	0.48
Citrus pulp	3.67	3.67	-	-
Rumensin, mg/d <sup>1</sup>	0	336.9	0	449.7
Other	8.96	8.96	5.19	5.19

Table 4. The nutrient profile (analyzed by NIR of weekly samples of fresh TMR) of the diets (mean  $\pm$  SD).

Item	Prepartum		Postpartum	
	CON	BMR	CON	BMR
<b>Analyses, % of ration DM</b>				
aNDFom	44.0 $\pm$ 3.2	41.6 $\pm$ 1.5	33.2 $\pm$ 2.0	32.4 $\pm$ 1.7
ADF	29.5 $\pm$ 1.8	27.2 $\pm$ 1.2	21.9 $\pm$ 1.7	20.7 $\pm$ 1.4
Starch	20.3 $\pm$ 2.1	21.1 $\pm$ 1.0	27.0 $\pm$ 1.1	26.7 $\pm$ 1.8
Sugar	4.7 $\pm$ 0.6	5.4 $\pm$ 0.4	4.0 $\pm$ 0.9	4.2 $\pm$ 1.0
Fat	2.7 $\pm$ 0.2	2.8 $\pm$ 0.2	4.3 $\pm$ 0.4	4.4 $\pm$ 0.3
uNDF <sub>240</sub>	17.1 $\pm$ 1.9	14.6 $\pm$ 1.2	11.2 $\pm$ 1.1	10.1 $\pm$ 1.1
peNDF <sup>1</sup>	35.1	35.3	22.8	23.4
MP, g/kg DM <sup>1</sup>	94.5	96.5	115.5	117.2

<sup>1</sup> Formulated value given by Cornell Net Carbohydrate and Protein System v. 6.5 using actual mean intakes

Intake and performance results are presented in Table 5 and Figure 4. In the prepartum period, DMI was higher for cows fed BMR than those fed CON ( $P=0.03$ ) corn silage, while cows fed RUM had lower intake than cows without RUM supplementation ( $P<0.01$ ). There were no significant interactions between source of corn silage and Rumensin. Postpartum there were no significant differences in intake due to corn silage, Rum, or any interaction. Milk yield however, was higher for cows fed BMR compared to cows fed CON corn silage ( $P=0.05$ ). There was also a three way interaction of corn silage, Rum, and Time where BMR-RUM had higher milk yield compared to CON-NO cows in weeks 5 and 6 postpartum ( $P=0.02$ ), these data are in Figure 4.

Table 5. Main effect means for prepartum and postpartum intake and milk yield.

Item	Corn Silage			Rumensin			P-Value		
	CON	BMR	SEM	NO	RUM	SEM	Corn	Rum	CxRxT <sup>1</sup>
<b>Prepartum</b>									
Intake, kg/d	14.0	14.7	0.21	14.5	13.9	0.21	0.03	<0.01	0.85
<b>Postpartum</b>									
Intake, kg/d	23.0	23.3	0.39	23.3	23.0	0.39	0.57	0.55	0.44
Yield, kg/d	45.8	48.3	0.86	46.7	47.4	0.86	0.05	0.61	0.02

<sup>1</sup>Interaction of Corn silage  $\times$  Rumensin  $\times$  Time, no other interactions for these variables were significant.

Prepartum and postpartum blood metabolite results are in Table 6. In the prepartum period cows fed BMR had lower NEFA than cows fed CON ( $P=0.02$ ), and cows fed RUM had lower BHBA than cows without Rumensin ( $P=0.04$ ). In the postpartum period cows fed BMR had lower NEFA and BHBA ( $P<0.01$ ) than cows fed CON corn silage. Cows fed RUM tended to have lower NEFA ( $P=0.06$ ), and had lower BHBA than cows without Rumensin. There were no interactions of corn silage type and Rumensin supplementation in either period.

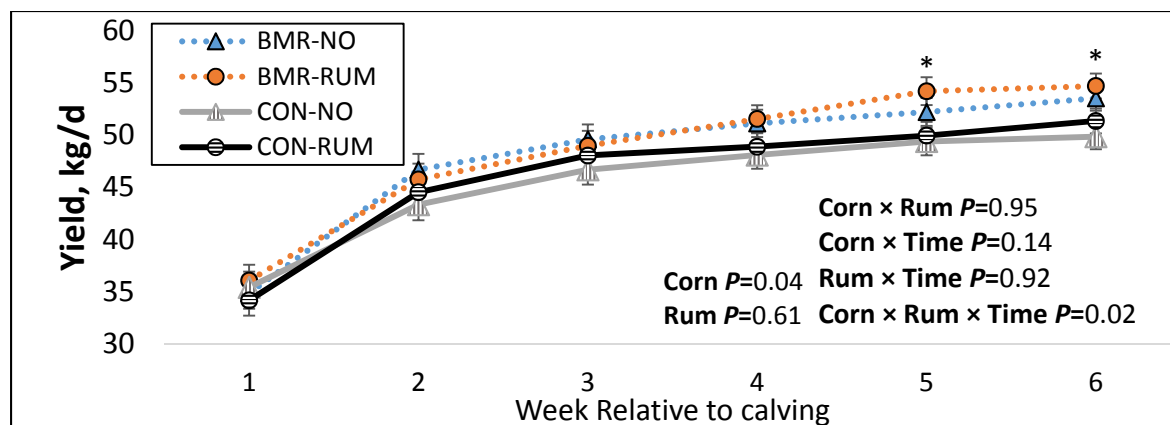


Figure 4. Milk yield by week relative to calving for all treatment groups.

Table 6. Prepartum and postpartum NEFA and BHBA presented as geometric means with back transformed 95% confidence limits.

Item	Corn		Rumensin		P-Value		
	CON	BMR	NO	RUM	Corn	Rum	CxT <sup>1</sup>
<b>Prepartum</b>							
NEFA, $\mu$ Eq/L	110.6 101.0-121.3	94.8 86.2-104.2	99.3 90.4-109.1	105.6 96.3-115.8	0.02	0.35	0.05
BHBA, mmol/L	0.72 0.68-0.77	0.70 0.66-0.75	0.75 0.70-0.79	0.68 0.64-0.72	0.62	0.04	0.53
<b>Postpartum</b>							
NEFA, $\mu$ Eq/L	458.9 415.7-506.7	369.1 334.2-407.7	440.0 398.4-486.0	385.0 348.7-425.0	<0.01	0.06	0.48
BHBA, mmol/L	1.22 1.10-1.35	1.00 0.90-1.11	1.21 1.10-1.34	1.00 0.91-1.11	<0.01	0.01	<0.01

<sup>1</sup>Interaction of Corn  $\times$  time, no other interactions for these variables were significant.

Cows fed BMR corn silage performed better overall than cows fed conventional corn silage through the transition period. Higher intakes prepartum, as well as higher yield and more favorable circulating blood metabolites in the postpartum period would suggest these cows were in a more positive energy balance than cows fed CON corn silage, likely due to the increased digestible fiber. With the greater digestibility, it is likely that microbial yield was greater, thus cows fed BMR also had greater MP supply and this could also have a positive impact on milk yield and cow health. Cows fed Rumensin, despite having slightly lower intakes prepartum, had lower circulating BHBA prepartum, as well as lower BHBA and NEFA in the postpartum period. This would again suggest that cows were in better metabolic status. Although we saw minimal statistical interactions between corn silage type and Rumensin, both use different strategies to increase the overall energy availability of the cow. In this vital transition period, these strategies, alone or together, can be key to increasing overall cow health and productivity through their impacts on energy availability and overall energy balance.

## CONCLUSIONS

The periparturient period is a pivotal time for dairy cows, and improving energy balance early after calving can greatly impact performance throughout lactation. Data presented here would suggest that uNDF<sub>240</sub> plays a role in regulating intake early in the fresh period, while feeding a highly digestible fiber ration improves metabolism and performance. Furthermore, use of corn silage hybrids with higher NDF digestibility such as BMR corn silage and addition of monensin improve performance and metabolic status. More research is needed to further investigate the interaction of fiber fractions and carbohydrates and the overall impacts on animal health and productivity. It is likely not just related to energy and there are some indications that MP balance is enhanced through the increased digestibility and intake and this likely has an impact on improving animal productivity. Understanding and utilizing different fiber fractions in fresh cow ration formulation could be key to improving intake, nutrient balance, overall health and milk production.

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## MOVING FORWARD WITH SILAGE HYBRID EVALUATION

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Our corn hybrid evaluation program for corn silage at Penn State began in 2001 to develop a corn hybrid evaluation program with the objective of meeting industry and producer needs. We developed a partnership with the Professional Dairy Managers of Pennsylvania and initiated an advisory team that consisted of seed industry representatives, dairy nutritionists, and dairy producers. We also have partnered with a commercial laboratory to provide their perspective on forage analysis techniques. Currently that laboratory is Cumberland Valley Analytical Services in Waynesboro Pennsylvania. One of the challenges we have encountered is the lack of consistency in the industry in the evaluation of corn silage quality and interpretation. In this presentation, we will discuss some of the foundations of our program and the directions for the future.

One objective of our program is to provide data from multiple environments over multiple maturity zones that exist in our state. Currently we have three maturity zones with at least three locations in each zone. We also strive to evaluate hybrids under the conditions that they are grown under. For us, this is often manured, following corn and no-till planting on dairy farms. At times this is challenging because these are often not the most uniform conditions. When we report our data, we separate hybrids into submaturity groups in each test and then sort by dry matter content at harvest. We encourage users to compare hybrids within these submaturity groups. This helps to reduce the impact of maturity on hybrid selection.

Our basic report has included yield and dry matter along with NIR based crude protein, NDF, NDFD (24 Hour) Starch, NEL, lignin, fat, and ash. For evaluation of BMR hybrids, we use wet chemistry based NDFD since we have found a better discrimination among BMR genes with the wet chemistry and approach and this has been confirmed by our laboratory partner. As we move forward we are making some changes in our program and have started to include some of the concepts addressed below.

In the past several years we have engaged with our advisory board to evaluate some new directions in corn silage evaluation. In addition we have engaged in discussions with other testing programs in the northeast at Cornell, Miner Institute, Western New York Crop Management Association and the University of Vermont. We have agreed to work together to develop new approaches together and strive for more consistency in our approach to corn silage evaluation. Based on these interactions we have focused on several areas to try to move forward with our forage analyses and interpretation. These included advanced fiber digestibility measurements, starch digestibility, and use of improved methods for interpretation of yield and quality data.

Several research efforts have suggested that evaluation of multiple time points of fiber digestibility could provide more insight into intake differences among forages and would be a better predictor of dry matter intake than NDFD. This has resulted in more laboratories offering a measurement of undigestible NDFD or uNDF that is based on the residue remaining after a 240 hour incubation and have developed NIR equations to predict this variable. Some laboratories have shown a large range in uNDF in corn silage samples and this is being incorporated as an input into dynamic dairy nutrition models such as CNCPS. Because of this need, our group has decided to incorporate uNDF in our evaluations. Our initial evaluations suggest that there are differences due to hybrid and environment due to uNDF and that there can be a significant range of for uNDF at a given NDFD level.

We have also been encouraged to evaluate starch digestibility differences among hybrids. We know that other factors such as particle size, moisture, the time in the silo can all have a significant effect. Our team has been working to identify the appropriate grind size to achieve the best repeatability to sort out hybrid differences to better reflect in situ starch digestibility. This year we plan to evaluate starch digestibility in several of our trials with a 1mm grind based on the results from a preliminary study conducted during the past two years. This will provide us with some replicated data across a range of hybrids in three different tests.

Developing better methods for interpreting silage yield and quality data is needed. Our advisory group has expressed concern about the use of Milk 2006 as a tool to predict potential milk production or profitability. A key concern is its sensitivity to fiber digestibility and may not fully account for improved intake and resulting milk production from some hybrids. Also, it is not possible to incorporate uNDF measurements in the prediction.

An alternative method for predicting milk response would be to use a dynamic rumen model such as CNCPS 6.5.5 to estimate potential milk differences among hybrids. Our Cornell partners used this approach in their program in 2016. They calculated estimated ME allowable milk yields for each hybrid using either a standard dry matter intake or a dry matter intake based on the uNDF240. They used a base ration with 28 lbs DM of corn silage and replaced each hybrid into the ration to calculate the individual values. When intakes were based on the uNDFD240, this resulted in potential milk differences among the hybrids up to nearly 20 lbs/day at one location. Our assessment is that these differences in estimated milk production seems a bit high and perhaps the model needs to be recalibrated. Nevertheless, we think the approach has merit and look forward to evaluating it in the future. Our hope is that a streamlined approach can be developed, based on model output, that can be used in a spreadsheet format.

This illustrates another goal of our programs which is not just to compare hybrids but to work together to develop approaches for hybrid testing that can be adopted by seed companies and others interested in evaluating corn hybrids for silage. Another objective is to use our programs as a platform for evaluations of other characteristics of

corn silage across hybrids. We are completing a study of fatty acid composition of commercial hybrids as one example. Cornell will be evaluating mycotoxin differences in a satellite study this year. We both hope to study environmental effects on hybrid performance more in the future and if we have some similarities in our protocols, we should be able to combine data across more environments than we were able to in the past.

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Cornell Corn Silage Variety Trials: <https://scs.cals.cornell.edu/extension-outreach/field-crop-production/variety-trials#corn-silage>



## IT'S THE COMBINATION: SCIENTIFIC DATA REVIEW OF THE FIRST CORN SILAGE TO BRING TOGETHER FIBER AND STARCH DIGESTIBILITY

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### INTRODUCTION

Lactating dairy cows require highly digestible forages with the correct mix of fermentable fiber and non-fiber carbohydrates to ensure efficient rumen fermentation, optimal feed intake, and high output of milk solids. With lower fiber digestibility, rumen fill constraints may limit dry matter intake (DMI) and productive capabilities of dairy cattle fed higher forage diets (Allen, 2000). Brown midrib (*bm3*) corn silage contains lower lignin content than conventional corn silage, resulting in improved NDF digestibility and greater DMI before rumen fill limitations are encountered (Ebling and Kung, 2004). But, starch digestibility is often lower for diets containing *bm3* rather than conventional corn silage. For example, Ferraretto and Shaver (2015) measured a 6%-unit reduction in rumen starch digestibility and a 1.4%-unit reduction in total tract starch digestibility for cows fed *bm3* versus conventional corn silage. The lower dietary starch digestibility with *bm3* corn silage may be related to: 1) greater passage rate of digesta driven by higher DMI (Moharrery et al., 2014), and(or) 2) greater kernel vitreousness of *bm3* corn hybrids compared with other commercially available corn hybrids (Ferraretto and Shaver, 2015).

Corn hybrids with floury kernel mutations, such as the *FL2* allele, contain less zein protein in the kernel and have greater rumen and total tract starch digestibility (Lopes et al., 2009). Zein protein cross-links and surrounds starch granules in the endosperm, preventing rumen microbial degradation of starch (Hoffman et al., 2011; Giuberti et al., 2014). Taylor and Allen (2005b) reported a 63% improvement in rumen starch digestion when diets contained floury versus vitreous corn grain. To-date, similar responses have not been observed for corn harvested at silage, rather than grain, moisture content, although Longuski et al. (2002) did observe a trend toward greater efficiency of fat-corrected milk production with floury endosperm corn silage.

The objective of this study was to compare diets containing a novel *bm3* corn silage hybrid with softer endosperm to diets containing commercially available conventional and *bm3* corn silage hybrids for their short-term effects on: 1) DMI, 2) lactational performance, 3) feeding behavior, and 4) total tract nutrient digestibility when fed to lactating Holstein cows. We hypothesized that the experimental *bm3* corn silage hybrid with softer endosperm would supply more fermentable carbohydrates (i.e., NDF and starch) than the conventional or *bm3* hybrids, resulting in greater milk component yields and gross feed efficiency.

## SILAGE TREATMENTS AND EXPERIMENTAL APPROACH

### Corn Silage Production

Three corn hybrids (Mycogen TMF2R447, 98-d relative maturity; Mycogen F2F498, 99-d relative maturity; and Mycogen FBDAS3, 96-d relative maturity, Mycogen Seeds, Dow AgroSciences LLC, Indianapolis, IN) were planted in a 6.07-ha field at the William H. Miner Agricultural Research Institute (Chazy, NY) on May 31, 2014 with 76-cm row spacing and 84,000 seeds/ha. Plots were separated by a buffer strip of a commercially available corn silage hybrid (Mycogen 2H079, 79-d relative maturity). All 3 plots were managed under the same tillage, fertilizer, and weed control procedures. Areas harvested were 1.32, 1.38, and 2.10 ha for TMF2R447, F2F498, and FBDAS3, respectively. Field plots were harvested using a self-propelled forage harvester with a kernel-processing unit (model 7300, John Deere, Moline, IL) with a 20.3-cm cut height, a 19-mm theoretical length of cut, and 3-mm roller gap spacing. Plots were harvested when kernel maturity reached ¼-milk line on October 7, 2014. Each corn silage hybrid was inoculated with Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) and stored in separate 2.7- × 30.5-m silage bags (AG-BAG plastic, Cottage Grove, MN). Average yields were 13.0, 13.8, and 11.7 tons of DM/ha for the TMF2R447, F2F498, and FBDAS3 corn silage hybrids, respectively.

### Experimental Design, Diets, Management of Cows, and Measurements

Fifteen multiparous Holstein cows were blocked by 3.5% fat-corrected milk and assigned randomly to 1 of 3 squares using a replicated 3 × 3 Latin square design with 28-d periods. Cows averaged (mean ± standard deviation) 682 ± 59 kg of BW, 112 ± 13 days in milk, and 2.4 ± 0.7 lactations at the start of the experiment. Cows were fed a diet containing 49.1% corn silage with the three diets formulated with a 1:1 replacement of corn silage: 1) conventional TMF2R447 (CCS); 2) F2F498 *bm3* (*BM3*); and 3) experimental FBDAS3 *bm3* hybrid with softer endosperm kernel genetics (BMR-EXP). Ingredient composition of the three dietary treatments is shown in Table 1. Diets were formulated with the Nutritional Dynamic System model (RUM&N Sas; Via Sant'Ambrogio, Italy) utilizing the Cornell Net Carbohydrate and Protein System (version 6.5). Diets were formulated using the description for a 3<sup>rd</sup> lactation cow, 119 days in milk with a BCS of 3.0, mature BW of 726 kg, and milk yield of 45 kg/d containing 3.8% fat and 3.2% true protein.

Cows were housed in a tie-stall barn on mattresses bedded with sawdust with individual feed boxes and water bowls. Cows were fed for ad libitum intake (targeted refusal rate of 10%) once daily at 1300 h; feed was pushed up at 0730 h. The diets were mixed in a Calan Data Ranger (American Calan, Inc., Northwood, NH). Cows were milked 3 times daily (0430, 1230, and 2030 h) in a double-12 parallel milking parlor (Xpressway Parallel Stall System; BouMatic, Madison, WI).

Table 1. Ingredient composition (% of DM) of the diets.

Item	Diets <sup>1</sup>		
	CCS	BM3	BMR-EXP
TMF2R447 corn silage	48.99	–	–
F2F498 corn silage	–	48.99	–
FBDAS3 corn silage	–	–	48.99
Hay crop silage	6.32	6.32	6.32
Grain mix			
Ground corn grain	13.91	13.91	13.91
Beet pulp pellets	7.26	7.26	7.26
Canola meal	6.41	6.41	6.41
Soybean meal, solvent extracted	5.69	5.69	5.69
Soybean meal, heat-treated <sup>2</sup>	4.17	4.17	4.17
Rumen inert fat <sup>3</sup>	2.13	2.13	2.13
Molasses	1.15	1.15	1.15
Blood meal	1.07	1.07	1.07
Calcium carbonate	0.99	0.99	0.99
Sodium sesquicarbonate <sup>4</sup>	0.79	0.79	0.79
Salt	0.39	0.39	0.39
Magnesium oxide	0.28	0.28	0.28
Urea	0.19	0.19	0.19
Methionine <sup>5</sup>	0.11	0.11	0.11
Yeast and organic selenium <sup>6</sup>	0.09	0.09	0.09
Vitamins A, D, and E <sup>7</sup>	0.03	0.03	0.03
Organic minerals <sup>8</sup>	0.01	0.01	0.01
Vitamin E <sup>9</sup>	0.01	0.01	0.01
Monensin <sup>10</sup>	0.01	0.01	0.01

<sup>1</sup>CCS = diet containing TMF2R447, a conventional corn silage; *BM3* = diet containing F2F498, a *bm3* corn silage; BMR-EXP = diet containing FBDAS3, a *bm3* corn silage with softer endosperm.

<sup>2</sup>Amino Plus; Ag. Processing, Inc.; Omaha, NE.

<sup>3</sup>BergaFat; Berg + Schmidt America, LLC; Libertyville, IL.

<sup>4</sup>S-Carb; FCM Industrial Chemical Group; Philadelphia, PA.

<sup>5</sup>Meta Smart; Adisseo USA, Inc.; Alpharetta, GA.

<sup>6</sup>Diamune; Diamond V Mills, Inc; Cedar Rapids, IA.

<sup>7</sup>Contained 24,093 kIU vitamin A/kg, 5,552 kIU vitamin D/kg, 92,676 IU vitamin E/kg.

<sup>8</sup>Availa 4; Zinpro Co.; Eden Prairie, MN.

<sup>9</sup>Contained 89,985 IU vitamin E/kg.

<sup>10</sup>Elanco Animal Health; Greenfield, IN.

Individual feed ingredients were collected weekly and dried in a forced-air oven at 105°C for 16 to 24 h for DM determination. Diets were adjusted for changes in the DM content of the feed ingredients when the DM of an ingredient varied by 1.2 standard deviations from the mean DM. Feed ingredients, diets, and orts were collected daily on d 22 to 28 of each period and dried in a forced-air oven at 105°C for 16 to 24 h for DM determination. Composites of feed ingredients, diets, and orts were analyzed for chemical composition by a commercial laboratory (Cumberland Valley Analytical

Services, Inc., Hagerstown, MD). Analyses included DM, ash (method 942.05; AOAC, 2012), OM (method 942.05; AOAC, 2012), CP (method 990.03; AOAC, 2012), soluble protein according to Krishnamoorthy et al. (1982), fat (method 2003.05; AOAC, 2012), ADF (method 973.18; AOAC, 2012), NDF using  $\alpha$ -amylase but excluding sodium sulfite (Van Soest et al., 1991), ADL (Goering and Van Soest, 1970), starch according to Hall (2009), sugar as ethanol soluble carbohydrates according to Dubois et al. (1956), and minerals (method 985.01; AOAC, 2012). Fermentation analysis was performed on the ensiled forage composite samples (Cumberland Valley Analytical Services, Inc.). Forage and diet composite samples were used to determine particle size distribution on an as-fed basis using the Penn State Particle Separator (Lammers et al., 1996) with 19-, 8-, and 4-mm screens. Particle size distribution was also determined on forages, the grain mix, and diets on a DM basis (55°C) by dry vertical sieving (Ro-Tap testing sieve shaker model B; W. S. Tyler Combustion Engineering, Inc., Mentor, OH) with 19.0-, 13.2-, 9.5-, 6.7-, 4.75-, 3.35-, 2.36-, 1.18-, 0.60-, and 0.30-mm sieves for 10 min. The physical effectiveness factor (pef) was determined by the standard dry vertical sieving method for feed ingredients and diets (Mertens, 2002).

In vitro NDF digestibility (30, 120, and 240 h) for forage composite samples (1-mm grind; Wiley mill, Arthur H. Thomas, Philadelphia, PA) were determined using an in vitro fermentation (Daisy<sup>II</sup> Incubator, Ankom Technology Corp., Fairpoint, NY) in buffered medium containing rumen fluid (Goering and Van Soest, 1970). Rumen in vitro digestibility of starch (7-h incubation) of grain mixes, diets, and (2- and 7-h incubation) corn silage ground to pass a 4-mm screen using a Wiley mill was determined (Cumberland Valley Analytical Services Inc.) according to Richards et al. (1995). Additionally, all 3 corn silage hybrids were analyzed for the fast pool rate (kd per h) of nutrient digestion, slow pool rate (kd per h) of nutrient digestion, the carbohydrate B<sub>1</sub> and B<sub>3</sub> rates (%/h) specific to the Cornell Net Carbohydrate and Protein System, and microbial biomass production (mg/g; Fermentrics; RFS Technologies, Ottawa, ON) according to Fermentrics Interpretation and Guidelines (2013).

Body weight was measured (Allweigh computerized scale; Allweigh Scale System Inc., Red Deer, AB) and BCS was assigned in 0.25-unit increments on a 1 to 5 scale (Ferguson et al., 1994) for each cow after the 1230 h milking at the beginning of the experiment and on d 28 of each period. Dry matter intake was determined by recording feed offered and refused daily on d 22 to 28 for each cow during each period. Dry matter content was determined for diets and orts on d 22 to 28 and used to calculate DMI. Milk yields were recorded (ProVantage Information Management System; Bou-Matic, Madison, WI) at every milking from d 22 to 28 of each period and used to calculate average daily yield. Milk samples from 3 consecutive milkings per d for each cow were collected on d 25 and 26 of each test period, preserved (Bronolab-W II Liquid Preservative; D & F Control Systems, Inc., Dublin, CA), and stored at 4°C. Milk samples were sent to a commercial laboratory (Dairy One, Ithaca, NY), and analyzed for fat, true protein, lactose, SNF, MUN (method 972.16; AOAC, 2012), and SCC by infrared procedures (Foss 4000; Foss Technology, Eden Prairie, MN). Feed efficiency (kg/kg) was calculated and expressed as actual milk/DMI, 3.5% FCM/DMI, and

SCM/DMI for d 25 through 26 of each period. Milk N efficiency was calculated as (kg of milk N/kg of N intake) × 100.

Cows were monitored for eating, rumination, and chewing (eating + rumination) behavior every 5 min for 2 consecutive 24-h blocks (d 25- 26) each period. Apparent total tract nutrient digestibility was determined from fecal grab samples collected on d 25 to 28 of each period so that every 3 h in a 24-h time period were represented. Composite samples of feces (by cow and period) were analyzed for DM, NDF, starch, and OM as described above. Undigested ash-free NDF (uNDFom) determined after a 240-h rumen in vitro incubation was used as an internal marker in the diets, orts, and feces and total tract digestibility was determined according to (Maynard et al., 1979).

Statistical analysis was performed using SAS (Version 9.2, SAS Institute Inc., Cary, NC). Data from the analysis of feed ingredients and diets were analyzed using the MEANS procedure of SAS (n = 3 per feed ingredient and diet). The data were reported as descriptive statistics (mean ± standard error). The experiment was conducted and analyzed as a Latin square design. Data collected over time (i.e., DMI, milk yield and composition, and behavior) were reduced to a period mean per cow. Data were subjected to ANOVA using the MIXED procedure of SAS. Fixed effects were treatment, period, square, and the treatment × square interaction. Cow nested within square was considered a random effect. Least squares means from the ANOVA results were separated using the Tukey procedure when the resulting *F*-test was  $P \leq 0.05$ . Significance was declared at  $P \leq 0.05$  and tendencies at  $0.05 < P \leq 0.10$ .

## RESULTS AND DISCUSSION

### Dietary and Ingredient Nutrient Composition

The NDF content averaged 42.9, 40.6, and 39.7% for CCS, *BM3*, and BMR-EXP, respectively (Table 2). The particle size distributions of all three corn silages were similar when assessed by the Penn State Particle Separator (as-fed basis) and Ro-Tap shaker method (DM-basis; data not shown).

Starch content averaged 30.2, 30.2, and 32.2% for CCS, *BM3*, and BMR-EXP, respectively. As expected, uNDFom was greater for CCS compared with *BM3* and BMR-EXP resulting in higher NDF digestibility for *BM3* and BMR-EXP compared with CCS. Digestibility of starch determined after a 2- and 7-h rumen in vitro incubation was similar among corn silage hybrids, averaging 28.5% and 80.1%, respectively. With Fermentrics analysis, the fast pool of nutrient digestion was 25.1, 37.7, and 36.7 %/h for the CCS, *BM3*, and BMR-EXP corn silage hybrids, respectively. With this system, the fast pool digestion rate is derived from the maximal *K*<sub>d</sub> per hour of silage acids, sugars, rapidly degraded starch, soluble fiber, and very rapidly digesting NDF (Fermentrics, 2013). Although there was little difference among the corn silage hybrids in starch digestion rate (B1 fraction), the rates increased slightly from CCS to *BM3* to BMR-EXP.

Table 2. Chemical composition, in vitro digestibility, and fermentation analysis (mean  $\pm$  standard error) of ingredients used in the diets<sup>1</sup>.

Item	CCS	<i>BM3</i>	BMR-EXP	Haycrop silage
DM, %	31.7 $\pm$ 0.3	28.7 $\pm$ 0.2	30.9 $\pm$ 0.2	34.1 $\pm$ 1.4
CP, % of DM	8.0 $\pm$ 0.0	7.9 $\pm$ 0.1	7.6 $\pm$ 0.1	15.1 $\pm$ 0.4
NDF <sup>2</sup> , % of DM	42.9 $\pm$ 1.0	40.6 $\pm$ 0.4	39.7 $\pm$ 1.6	55.7 $\pm$ 1.0
ADL, % of DM	3.2 $\pm$ 0.3	2.0 $\pm$ 0.2	2.0 $\pm$ 0.2	5.4 $\pm$ 0.0
Starch, % of DM	30.2 $\pm$ 0.5	30.2 $\pm$ 0.3	32.2 $\pm$ 1.3	1.9 $\pm$ 0.1
ESC <sup>3</sup> , % of DM	1.2 $\pm$ 0.0	1.4 $\pm$ 0.0	1.5 $\pm$ 0.1	3.3 $\pm$ 0.4
Fat, % of DM	3.0 $\pm$ 0.0	3.2 $\pm$ 0.0	3.2 $\pm$ 0.0	4.2 $\pm$ 0.4
Ash, % of DM	4.5 $\pm$ 0.3	4.3 $\pm$ 0.2	4.03 $\pm$ 0.3	9.5 $\pm$ 0.5
Lactic acid, % of DM	4.0 $\pm$ 0.2	4.4 $\pm$ 0.2	3.8 $\pm$ 0.4	4.5 $\pm$ 0.4
Acetic acid, % of DM	1.5 $\pm$ 0.2	1.8 $\pm$ 0.1	2.3 $\pm$ 0.2	2.6 $\pm$ 0.2
Total VFA, % of DM	5.5 $\pm$ 0.3	6.2 $\pm$ 0.2	6.1 $\pm$ 0.2	7.9 $\pm$ 0.0
Ammonia, % of CP	0.7 $\pm$ 0.0	0.6 $\pm$ 0.0	1.1 $\pm$ 0.5	1.9 $\pm$ 0.6
pH	3.9 $\pm$ 0.1	3.8 $\pm$ 0.0	3.8 $\pm$ 0.0	4.5 $\pm$ 0.1
30-h uNDFom, % of DM <sup>4</sup>	23.6 $\pm$ 0.3	17.3 $\pm$ 0.5	16.3 $\pm$ 0.7	32.1 $\pm$ 1.7
120-h uNDFom, % of DM	13.9 $\pm$ 0.2	8.7 $\pm$ 0.3	7.7 $\pm$ 0.3	22.6 $\pm$ 1.3
240-h uNDFom, % of DM	12.6 $\pm$ 0.2	6.8 $\pm$ 0.2	6.6 $\pm$ 0.3	19.5 $\pm$ 2.5
2-h starch digestibility, % of starch	28.1 $\pm$ 4.05	27.9 $\pm$ 3.8	29.4 $\pm$ 2.5	—
7-h starch digestibility, % of starch	81.0 $\pm$ 3.2	79.7 $\pm$ 4.7	79.6 $\pm$ 3.9	—
Fast pool Kd, %/h <sup>5</sup>	25.1 $\pm$ 4.1	37.7 $\pm$ 8.0	36.7 $\pm$ 4.1	—
Slow pool Kd, %/h	3.0 $\pm$ 0.6	3.8 $\pm$ 1.0	3.0 $\pm$ 0.3	—
Carbohydrate B <sub>1</sub> , %/h	20.3 $\pm$ 0.6	21.8 $\pm$ 1.9	22.9 $\pm$ 0.7	—
Carbohydrate B <sub>3</sub> , %/h	3.0 $\pm$ 0.6	3.8 $\pm$ 1.0	3.0 $\pm$ 0.3	—
Microbial biomass, mg/g	125.2 $\pm$ 3.7	138.3 $\pm$ 3.8	147.0 $\pm$ 5.6	—

<sup>1</sup>CCS = TMF2R447, a conventional corn silage; *BM3* = F2F498, a *bm3* corn silage; BMR-EXP = FBDAS3, a *bm3* corn silage with softer endosperm.

<sup>2</sup>NDF with residual ash using  $\alpha$ -amylase without sodium sulfite.

<sup>3</sup>ESC = Ethanol soluble carbohydrate.

<sup>4</sup>uNDFom = undigested NDF on organic matter basis (i.e., ash-free).

<sup>5</sup>Fast pool, slow pool, carbohydrate B<sub>1</sub> and B<sub>3</sub> rate, and microbial biomass were analyzed by Fermentrics (RFS Technologies, Ottawa, ON).

Microbial biomass production, which is considered to be the “gold standard” parameter in this system and found to be associated with higher milk yield, was greater for the BMR-EXP corn silage hybrid (147.0 mg/g) compared to CCS (125.2 mg/g) and *BM3* (138.3 mg/g). These differences reflect the potential for greater rumen carbohydrate digestibility and increased microbial protein synthesis for BMR-EXP. The daily microbial biomass production (MBP) can be calculated using the formula: Rumen microbial protein production (g/d) = MBP  $\times$  0.41  $\times$  1.30  $\times$  DMI (kg/d). With this formula, we predict microbial protein production of 1788, 2063, and 2163 g/d for CCS, *BM3*, and BMR-EXP, respectively. From a protein perspective, these differences translate into

approximately 2 kg/d more milk for BMR-EXP versus *BM3*, and 6 kg/d more milk for BMR-EXP versus CCS. These predicted differences in milk yield, driven by microbial biomass, agree reasonably well with the measured milk production shown in Table 5.

Chemical composition and in vitro NDF and starch digestibility of the diets are presented in Table 3. The content of CP, NDF, starch, and sugars were similar across the diets. Particle distribution (as-fed basis) based on the Penn State Particle Separator and particle size (DM basis) were consistent across all diets with an average physical effectiveness factor and peNDF value of 0.69 and 22.2%, respectively.

Table 3. Chemical composition (mean  $\pm$  standard error) of the diets.

Item	Diets <sup>1</sup>		
	CCS	<i>BM3</i>	BMR-EXP
DM, %	45.4 $\pm$ 0.4	41.3 $\pm$ 0.4	44.2 $\pm$ 0.2
CP, % of DM	15.1 $\pm$ 0.2	14.8 $\pm$ 0.1	14.6 $\pm$ 1.0
aNDF <sup>2</sup> , % DM	33.0 $\pm$ 1.1	32.6 $\pm$ 0.4	32.4 $\pm$ 1.3
Starch, % DM	26.1 $\pm$ 0.9	25.7 $\pm$ 0.6	27.1 $\pm$ 1.3
ESC <sup>3</sup> , % DM	4.4 $\pm$ 0.6	4.8 $\pm$ 0.7	5.1 $\pm$ 0.8
Fat, % DM	4.6 $\pm$ 0.0	4.5 $\pm$ 0.3	4.3 $\pm$ 0.2
Ash, % DM	7.1 $\pm$ 0.2	7.6 $\pm$ 0.7	6.9 $\pm$ 0.3

<sup>1</sup>CCS = diet containing TMF2R447, a conventional corn silage; *BM3* = diet containing F2F498, a *bm3* corn silage; BMR-EXP = diet containing FBDAS3, a *bm3* corn silage with softer endosperm.

<sup>2</sup> NDF with residual ash using  $\alpha$ -amylase without sodium sulfite.

<sup>3</sup> ESC = Ethanol soluble carbohydrate.

### Dry Matter and Nutrient Intake

Dry matter and nutrient intake, BW, and BCS are summarized in Table 4. Dry matter intake, expressed as kg/d and % of BW, was greater for cows consuming *BM3* (28.0 kg/d and 4.03%) compared with CCS (26.8 kg/d and 3.87%;  $P = 0.013$  and  $P = 0.005$ ). Dry matter intake for cows consuming BMR-EXP was intermediate (27.6 kg/d and 3.94%). Holt et al. (2013a) found that, for cows between 60 and 180 days in milk, those fed a *bm3* corn silage diet consumed approximately 1 kg/d more than cows fed a conventional corn silage diet, likely due to reduced gut fill for cows consuming *bm3*. Similarly, Ferraretto and Shaver (2015) reported a 0.9 kg/d increase in DMI for cows consuming brown midrib corn silages, most likely related to increased NDF digestibility and rumen passage rate. In contrast to fiber, Taylor and Allen (2005a) reported no effect of grain endosperm type on DMI in diets containing floury or vitreous endosperm corn.

Neutral detergent fiber intake was increased for cows consuming both *BM3* (9.2 kg/d;  $P = 0.009$ ) and BMR-EXP (9.2 kg/d;  $P = 0.003$ ) compared with CCS (8.7 kg/d). Taylor and Allen (2005b) observed no differences in NDF intake between cows fed diets containing conventional and *bm3* corn silage. Because undigested NDF intake was 0.7 kg/d greater ( $P = 0.001$ ) and potentially digestible NDF intake was 1.2 kg/d less ( $P =$

0.001) for cows consuming CCS compared with both brown midrib hybrids, the differences in NDF intake may be due to interactions between gut fill, passage rate, and NDF digestibility. Starch intake was increased for BMR-EXP (7.5 kg/d) compared with CCS (7.0 kg/d;  $P = 0.005$ ) while *BM3* was intermediate (7.2 kg/d).

Table 4. Least squares means of DMI, BW, and BCS of multiparous Holstein cows fed diets containing different sources of corn silage.

Item	Diets <sup>1</sup>			SEM	P-value
	CCS	<i>BM3</i>	BMR-EXP		
DMI, kg/d	26.8 <sup>b</sup>	28.0 <sup>a</sup>	27.6 <sup>ab</sup>	0.5	0.016
DMI, % of BW	3.87 <sup>b</sup>	4.03 <sup>a</sup>	3.94 <sup>ab</sup>	0.08	0.006
NDF intake, kg/d	8.7 <sup>b</sup>	9.2 <sup>a</sup>	9.2 <sup>a</sup>	0.16	0.002
uNDFom intake <sup>2</sup> , kg/d	2.3 <sup>a</sup>	1.6 <sup>b</sup>	1.6 <sup>b</sup>	0.03	0.001
pdNDF intake <sup>3</sup> , kg/d	6.4 <sup>b</sup>	7.6 <sup>a</sup>	7.6 <sup>a</sup>	0.14	0.001
Starch intake, kg/d	7.0 <sup>b</sup>	7.2 <sup>ab</sup>	7.5 <sup>a</sup>	0.17	0.007
BW, kg	696	698	705	16	0.21
BW change, kg/28-d period	8	5	20	4	0.08
BCS	2.86	2.78	2.84	0.08	0.18
BCS change, kg/28-d period	-0.04	0.05	-0.01	0.04	0.41

<sup>ab</sup>Least squares means within a row without a common superscript differ ( $P \leq 0.05$ ).

<sup>1</sup>CCS = diet containing TMF2R447, a conventional corn silage; *BM3* = diet containing F2F498, a *bm3* corn silage; BMR-EXP = diet containing FBDAS3, a *bm3* corn silage with softer endosperm.

<sup>2</sup>Undigested NDF, determined after a 240 h rumen in vitro incubation.

<sup>3</sup>Potentially digestible NDF = (NDF – uNDFom).

Body weight and BCS were unaffected by dietary treatment. Change in body weight (kg/28-d period) tended ( $P = 0.08$ ) to be greater for cows consuming the BMR-EXP diet (20 kg) compared with *BM3* (5 kg) or CCS (8 kg).

#### Lactational Performance

Milk yield, FCM, SCM, and ECM were greater for cows consuming *BM3* and BMR-EXP compared to cows that were fed CCS ( $P = 0.001$ ; Table 5). Increased milk yields have been reported when cows were fed 30 to 40% *bm3* corn silage-based diets in comparison to diets containing conventional corn silage (Lim et al., 2014; Ferraretto and Shaver, 2015). Milk fat content was greater for cows consuming CCS (4.0%) compared to *BM3* (3.85%;  $P = 0.02$ ) and BMR-EXP (3.87%;  $P = 0.04$ ), but milk fat yield was greatest for cows fed BMR-EXP (1.87 kg/d) compared to CCS (1.74 kg/d;  $P = 0.001$ ) and *BM3* (1.80 kg/d;  $P = 0.05$ ).



Table 5. Least squares means of lactational performance and feed efficiency for multiparous Holstein cows fed diets containing different sources of corn silage

Item	Diets <sup>1</sup>			SEM	P-value
	CCS	BM3	BMR-EXP		
Milk yield					
Milk, kg/d	43.8 <sup>b</sup>	47.3 <sup>a</sup>	48.0 <sup>a</sup>	1.3	0.001
3.5% FCM, kg/d	47.1 <sup>b</sup>	49.7 <sup>a</sup>	51.2 <sup>a</sup>	1.4	0.001
SCM, kg/d	43.1 <sup>b</sup>	46.2 <sup>a</sup>	47.8 <sup>a</sup>	1.4	0.001
ECM, kg/d	47.2 <sup>b</sup>	50.3 <sup>a</sup>	51.8 <sup>a</sup>	1.5	0.001
Milk composition					
Fat, %	4.00 <sup>a</sup>	3.85 <sup>b</sup>	3.87 <sup>b</sup>	0.07	0.013
Fat, kg/d	1.74 <sup>b</sup>	1.80 <sup>b</sup>	1.87 <sup>a</sup>	0.06	0.001
True protein, %	3.13 <sup>b</sup>	3.19 <sup>a</sup>	3.19 <sup>a</sup>	0.07	0.012
True protein, kg/d	1.36 <sup>b</sup>	1.49 <sup>a</sup>	1.54 <sup>a</sup>	0.06	0.001
Lactose, %	4.80 <sup>b</sup>	4.85 <sup>a</sup>	4.84 <sup>ab</sup>	0.03	0.012
Lactose, kg/d	2.08 <sup>b</sup>	2.28 <sup>a</sup>	2.34 <sup>a</sup>	0.07	0.001
SNF, %	8.80 <sup>b</sup>	8.93 <sup>a</sup>	8.93 <sup>a</sup>	0.06	0.001
SNF, kg/d	3.82 <sup>b</sup>	4.19 <sup>a</sup>	4.32 <sup>a</sup>	0.13	0.001
MUN, mg/dL	13.60 <sup>a</sup>	11.61 <sup>b</sup>	11.16 <sup>b</sup>	0.30	0.001
SCS	1.93	1.58	1.17	0.40	0.349
Feed efficiency					
Milk/DMI	1.63 <sup>b</sup>	1.69 <sup>ab</sup>	1.74 <sup>a</sup>	0.04	0.001
3.5% FCM/DMI	1.75 <sup>b</sup>	1.77 <sup>b</sup>	1.85 <sup>a</sup>	0.04	0.004
SCM/DMI	1.60 <sup>b</sup>	1.65 <sup>b</sup>	1.73 <sup>a</sup>	0.04	0.001
ECM/DMI	1.76 <sup>b</sup>	1.79 <sup>b</sup>	1.87 <sup>a</sup>	0.04	0.001
Milk N efficiency, %	35.3 <sup>c</sup>	38.1 <sup>b</sup>	40.4 <sup>a</sup>	1.2	0.001

<sup>abc</sup> Least squares means within a row without a common superscript differ ( $P \leq 0.05$ ).

<sup>1</sup>CCS = diet containing TMF2R447, a conventional corn silage; BM3 = diet containing F2F498, a *bm3* corn silage; BMR-EXP = diet containing FBDAS3, a *bm3* corn silage with softer endosperm.

Milk true protein content and yield were greater for cows fed BM3 and BMR-EXP compared to cows fed CCS ( $P < 0.03$  and  $P = 0.001$ , respectively). Lim et al. (2014) also reported increased protein content and yield for cows fed *bm3* corn silage diets compared to conventional corn silage diets. Differences in protein content and yield may be related to greater microbial protein synthesis due to increased rumen OM digestibility for cows fed *bm3* corn silage diets. When Ramirez et al. (2012) estimated microbial CP by measuring purine derivatives in diets containing 40% *bm3* or dual purpose corn silage, *bm3* corn silage diets resulted in greater calculated microbial CP flows. These results agree well with our study where the Fermentrics data showed greater microbial biomass production for the BM3 and particularly for the BMR-EXP corn silages.

Solids non-fat content and yield were greater for cows fed BM3 and BMR-EXP compared to cows fed CCS ( $P = 0.001$ ). Milk urea N concentration was greater for cows consuming CCS (13.60 mg/dL) compared to BM3 (11.61 mg/dL;  $P = 0.001$ ) and BMR-

EXP (11.16 mg/dL;  $P = 0.001$ ). Others have also reported a reduction in MUN concentration when feeding brown midrib silages (Taylor and Allen, 2005b; Holt et al., 2013b).

Feed efficiency (milk/DMI) was greatest for cows consuming BMR-EXP (1.74 kg milk/kg DMI) compared to cows consuming CCS (1.63 kg milk/kg DMI;  $P = 0.001$ ), and intermediate for cows consuming *BM3* (1.69 kg milk/kg DMI). Cows consuming BMR-EXP had greater efficiency of production of 3.5% FCM, SCM and ECM when compared to either the *BM3* or CCS-fed cows ( $P < 0.005$ ). Several studies have reported that feed efficiency is similar for cows fed *bm3* and conventional corn silage diets (Stone et al., 2012; Holt et al., 2013a; Ferraretto and Shaver, 2015). However, Taylor and Allen (2005a) reported that floury grain not originating in silage tended to increase 3.5% FCM, indicating that a source of more digestible floury grain in diets may improve feed efficiency.

Milk N efficiency was greatest for cows consuming BMR-EXP (40.4%), compared to cows consuming *BM3* (38.1%;  $P = 0.03$ ) and CCS (35.3%;  $P = 0.001$ ). Milk N efficiency was also greater for cows consuming *BM3* compared to CCS ( $P = 0.006$ ). Holt et al. (2013b) demonstrated that feeding forages greater in rumen degradability such as *bm3* corn silage resulted in improved N utilization. Our study indicates that the combined effects of greater rumen fiber degradability and greater total carbohydrate fermentability provided by feeding the BMR-EXP corn silage enhanced N utilization of dairy cows. The observed responses of greater N efficiency, greater ECM/DMI, lower MUN, and higher microbial biomass production all fit together.

## Feeding Behavior

Chewing, eating, and rumination behavior expressed as minutes per day were similar across all diets (Table 6). Interestingly, even though DMI was the least, chewing and ruminating time expressed as minutes per kilogram of DM was greater for cows fed the CCS diet compared with the *BM3* diet ( $P = 0.006$ ) but did not differ when compared to cows fed the BMR-EXP diet. Feeding behavior differences based on NDF and peNDF intake may better reflect biological differences among treatments. Chewing time per kilogram of NDF was increased for cows consuming CCS (96.5 min/kg NDF) compared with *BM3* and BMR-EXP (91 min/kg NDF;  $P = 0.002$ ). Forage fragility, while not incorporated into pef values, plays a role in chewing activity (Mertens, 1997). Differences in forage fragility of *BM3* and BMR-EXP may help account for decreased chewing time per kilogram of NDF which subsequently allowed for 0.5 kg/d greater NDF intake for cows consuming *bm3* hybrids because fiber containing lower lignin or uNDFom content requires less fracture force for exposure to microbial degradation. Eating time on an NDF basis was greater for cows consuming CCS (33.9 min/kg NDF) compared to cows consuming BMR-EXP (30.9 min/kg NDF;  $P = 0.03$ ) and intermediate for cows consuming *BM3* (32.3 min/kg NDF). Cows consuming CCS ruminated longer per kilogram of NDF than either group of cows fed *bm3* ( $P = 0.002$ ).

Table 6. Least squares means of eating and ruminating behavior for multiparous Holstein cows fed diets containing different sources of corn silage.

Item	Diets <sup>1</sup>			SEM	P-value
	CCS	BM3	BMR-EXP		
Chewing					
min/d	838	826	832	16	0.645
min/kg DM	31.4 <sup>a</sup>	29.7 <sup>b</sup>	30.3 <sup>ab</sup>	0.8	0.006
min/kg NDF	96.5 <sup>a</sup>	90.8 <sup>b</sup>	90.6 <sup>b</sup>	2.3	0.001
Eating					
min/d	294	294	284	11	0.41
min/kg DM	11.0	10.5	10.4	0.4	0.126
min/kg NDF	33.9 <sup>a</sup>	32.3 <sup>ab</sup>	30.9 <sup>b</sup>	1.3	0.027
Ruminating					
min/d	543	532	547	13	0.303
min/kg DM	20.4 <sup>a</sup>	19.1 <sup>b</sup>	19.9 <sup>ab</sup>	0.6	0.006
min/kg NDF	62.6 <sup>a</sup>	58.5 <sup>b</sup>	59.6 <sup>b</sup>	1.9	0.002

<sup>ab</sup>Least squares means within a row without a common superscript differ ( $P \leq 0.05$ ).

<sup>1</sup>CCS = diet containing TMF2R447, a conventional corn silage; BM3 = diet containing F2F498, a *bm3* corn silage; BMR-EXP = diet containing FBDAS3, a *bm3* corn silage with softer endosperm.

#### Total Tract Digestibility

Apparent total tract digestibility for all nutrients was unaffected by dietary treatment (Table 7). Total tract digestibility of OM, NDF, and potentially digestible NDF averaged 74.4, 58.1, and 73.0%, respectively. Ferraretto and Shaver (2015) reported that treatments containing *bm3* corn silage had greater total tract NDF digestibility (44.8%) compared to conventional corn silage (42.3%). In contrast, similar NDF digestibility was observed by Ferraretto et al. (2015) and thought to be related to greater passage rate of digesta due to higher DMI. Total tract starch digestibility was similar among treatments, averaging 99.3%.

Table 7. Least squares means of apparent total tract nutrient digestibility (%) of the diets for multiparous Holstein cows fed diets containing different corn silages.

Item	Diets <sup>1</sup>			SEM	P-value
	CCS	BM3	BMR-EXP		
OM	74.2	74.6	74.3	0.3	0.31
NDF	58.2	58.4	57.8	0.5	0.62
Potentially digestible NDF	73.0	73.0	72.9	0.5	0.98
Starch	99.3	99.4	99.3	0.1	0.42

<sup>1</sup> CCS = diet containing TMF2R447, a conventional corn silage; BM3 = diet containing F2F498, a *bm3* corn silage; BMR-EXP = diet containing FBDAS3, a *bm3* corn silage with softer endosperm.

## CONCLUSIONS

The results of this experiment show that an experimental *bm3* corn silage hybrid containing softer endosperm improves NDF intake similar to traditional *bm3* hybrids, while also increasing starch intake compared to conventional corn silage. Greater feed efficiency (FCM, SCM and ECM) indicates that the *bm3* corn silage hybrid containing softer endosperm improves energy utilization compared to *bm3* and conventional corn silage. Additionally, improved milk N efficiency suggests that greater rumen degradability and greater carbohydrate fermentability can be achieved when feeding a BMR-EXP diet. Availability of a *bm3* corn silage hybrid with softer endosperm will allow dairy producers to feed a high quality forage that will efficiently satisfy both fiber and energy requirements of high producing dairy cows.

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