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New Insights in Milk Fat Research and Reviewing Our Progress

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Introduction

Dairymen are largely paid for the pounds of milk fat and protein shipped. Milk fat is the most variable of milk's components and provides an opportunity to increase farm income. Milk fat concentration and yield is responsive to multiple dietary, genetic, and environmental factors. Diet-induced milk fat depression explains large decreases in milk fat that occur during disrupted rumen fermentation and was the predominant focus of milk fat research for many decades, especially after the discovery of bioactive conjugated linoleic acid isomers just over twenty years ago. A large number of dietary and environmental factors contribute to the risk for diet-induced milk fat depression including large contributions from dietary unsaturated fatty acids and fermentability. More recently, research has focused on other dietary and non-nutritional factors that impact milk fat yield. Importantly, these factors have broad application to allow small, but very economically significant increases in milk fat yield and profitability. For example, extensive work has been done demonstrating the efficacy of fat supplements and specifically characterizing the ability of palmitic acid to increase milk fat yield. Increasing acetate supply also increases milk fat yield. The seasonal variation in milk component concentration and yield has also been characterized and is important to setting goals and expectations. Milk fat is a highly heritable trait and large variation exists in genetic potential between cows within a herd. Although there does not appear to be much variation between herds, the average genetic potential has increased in recent years. Maximizing milk fat yield required a holistic approach that spans from nutrition to management and continues to evolve as we gain a better understanding of the impact of each factor.

What is "Milk Fat Depression (MFD)"

The term "milk fat depression" is a common term used by nutritionists and producers and has slightly different definitions between individuals. The classic reviews by Bauman and coworkers specifically discussed "classic diet-induced milk fat depression" that was defined as a decrease in milk fat associated with disrupted rumen fermentation (Griinari et al., 1998, Bauman and Griinari, 2003). It is important to note that this is a specific condition and not simply any change in milk fat yield. Phenotypically, up to a 50% reduction in milk fat concentration and yield can be observed with no decrease in milk or milk protein yield. Extensive work over the past 20 years has demonstrated that diet-induced MFD is caused by unique bioactive conjugated linoleic acid (CLA) isomers that are made during rumen biohydrogenation of unsaturated fatty acids (FA) by an altered rumen microbial community. Since diet-induced MFD is caused by these bioactive FA it can be most accurately called

biohydrogenation-induced MFD (BH-induced MFD). Investigating this condition has provided insight into the regulation of milk fat synthesis and management strategies to reduce inhibition of milk fat synthesis (Reviewed by Harvatine et al., 2009). Large decreases in milk fat (>15%) is almost undoubtably BH-induced MFD, but this mechanism does not explain many other smaller changes in milk fat synthesis. The occurrence of BH-induced MFD is best diagnosed by milk fat concentration of *trans*-10 C18:1, although this requires analysis by gas chromatography.

Variation in Milk Fat Between and Within Herds

Milk fat concentration and yield is variable between farms because of differences in diet, management practices, and herd genetics among other factors and demonstrates both challenges and opportunities. Milk fat averaged 3.73% [standard deviation (SD) = 0.33], but the 10^{th} and 90^{th} percentile were 3.34 to 4.12% in a database of Dairy Herd Improvement Association (DHIA) test days of Holstein herds in MN, PA, TX, and FL from 2004 to 2016 from the Dairy Records Management Database (http://www.drms.org/; Unpublished). There is also substantial variation in milk fat concentration and yield between cows within a farm.

The variation between cows and herds highlights the opportunity to increase milk fat. It is important to keep in mind that average milk fat can be increased by two different approaches. You can increase all cows a small amount, but it is probably difficult to increase cows who are already high in the distribution as they are at their genetic and physiological potential. Alternatively, the cows in the lower part of the distribution are likely below their genetic and physiological potential and interventions may be able to result in substantial increases (25+%). Large increases in these cows can result in an increase in the herd average.

Non-Nutritional Factors Impacting Milk Fat Yield

Genetics of Milk Fat Concentration and Yield

Milk fat concentration and yield are highly heritable [0.45 and 0.29, respectively; (Welper and Freeman, 1992)] and milk fat is unique in that the genetic variation is due to a limited number of single nucleotide polymorphisms (SNPs) with large individual effects (Hayes et al., 2010). The largest effect is a K232A SNP in diacylglycerol acyltransferase [DGAT1; (Grisart et al., 2002)] followed by the F279Y SNP in the growth hormone receptor [GHR; milk fat allele substitution effect 0.46 percentage units; (Signorelli et al., 2009)]. Wang et al. (2012) identified four quantitative trait loci that explained over 46% of the genetic variation in milk fat concentration including 34% explained by DGAT1 and 12% by GHR. We recently characterized the variation in predicted transmitting ability for fat production between nearly 6,000 herds available in the Dairy Records Management System database. Very little variation was observed between herds, although larger variation is commonly observed between cows within a herd. Importantly, average genetic potential has increased considerably over the past

decade due to changes in selection indexes and genomic selection and should be considered when evaluating if a farm is reaching its potential.

Annual Rhythms in the Dairy Cow

Rather than simply *responding* to a change in the environment after it occurs, time keeping mechanisms in the hypothalamus allow the animal to *anticipate* yearly environmental changes before they occur. Yearly patterns of milk production have been recognized for over 40 years (Wood, 1970). When examining average monthly bulk tank records from the United States Federal Milk Marketing Orders, the presence of an annual rhythm is apparent. These yearly patterns fit a robust cosine function, suggesting that they represent a biological rhythm (Salfer et al., 2019). The variation in milk fat concentration due to the annual rhythm is between 0.15 and 0.30 percentage units, depending on the region with a lower amplitude in southern regions of the United States. The presence of yearly production rhythms was confirmed using ten years of DHIA data from individual herds in Minnesota, Pennsylvania, Texas and Florida (Salfer et al., 2017). Although fat and protein concentration both peak near the first of the year, the annual rhythm of milk yield peaks between late March and early April, right around the vernal equinox (Salfer et al., 2017). Fat and protein yield peak between late February and early March. Contrary to the rhythms of fat and protein concentration, amplitudes of annual milk yield rhythms are greater in the southern U.S. compared to the north. Fat and protein yield also oscillated more in the southern U.S. than the northern U.S. Producers and nutritionists should change their goal for milk fat concentration and yield across the year and future work may provide insight into how to reduce the impact of the cycle on production.

Circadian Patterns of Milk Fat

Circadian rhythms are daily patterns and the dairy cow has a daily pattern of milk synthesis that impacts milk yield and composition. Generally, milk yield is highest in the morning, but milk fat concentration is higher in the evening (Gilbert et al., 1972, Quist et al., 2008). We have also observed milk yield and milk composition vary across the day while milking every 6 h in multiple experiments. The first consideration is that care needs to be taken in interpreting milk composition of a single milking. We have also observed the daily rhythms are dependent on the timing of feed intake and length of time without feed each day demonstrating the importance of feed management, including selecting feeding times and frequency, on milk production

Energy Balance and Stage of Lactation

Milk fat is very high at the initiation of lactation and rapidly decreases to a nadir at peak milk yield, and then gradually increases over the course of lactation. In early lactation, cows are in a negative energy balance and have high levels of fat being mobilized from adipose tissue. Mass action kinetics increase uptake of these fatty acids by the mammary gland as plasma non-esterified FA (NEFA) increase due to mobilization. It is not uncommon for milk fat concentrations to be above 5% in early

lactation cows, and very high levels are a poor indication of transition cow health. The increase in milk fat after peak is indicative of a shift in the ratio of milk fat to lactose synthesis and is expected to be partly due to endocrine regulation. Later lactation cows are also less susceptible to diet-induced MFD. It is important to consider days in milk when evaluating milk fat concentration of individual cows and groups.

Milk Flow

Milk fat concentration is top of mind for producers and nutritionists, but milk fat yield is what is economically important. Fat yield is influenced both by milk fat concentration and milk yield. First, care needs to be taken to not decrease milk yield when attempting to increase milk fat concentration. This is especially important considering that decreases in milk yield likely also decrease milk protein yield. Secondly, maximizing milk fat yield requires optimal production. Milk yield is under complex regulation with major influence from endocrine mechanisms and can be limited by nutritional, health, or environmental stressors. With this in mind, all good management practices that increase reproductive efficiency, cow health, cow comfort, etc. and increase level of milk production likely also increase milk fat yield.

Nutritional Factors Impacting Milk Fat Yield

Biohydrogenation Induced Milk Fat Depression

Diet-induced, or BH-induced MFD, is caused by disrupted rumen fermentation that results in a shift in the rumen microbial population. The specific causative microbes is not clear, but a decrease in microbial diversity is apparent in microbiome analysis (Pitta et al., 2018, Pitta et al., 2020). Prediction of the occurrence of BH-induced MFD is complex because it is not directly caused by a single dietary factor; rather it is the result of the interaction of numerous factors that reduce the rate of biohydrogenation and shift biohydrogenation to the alternate pathway. It is preferable to think of dietary risk factors that move a diet along a continuum from low to high risk. Extensive work has highlighted dietary factors that increase and decrease risk. Briefly, risk is increased by increasing diet fermentability and unsaturated fatty acids, decreasing effective fiber, ionophores, poorly fermented silages, slug feeding and other factors that decrease rumen pH or disturb normal rumen fermentation. Risk is decreased by increasing dietary cation-anion difference and feeding 2-hydroxy-4 (methylthio) butanoic acid (HMTBA) (Baldin et al., 2018, Baldin et al., 2019). Less direct data is available for direct fed microbial products, but good mechanisms exist to support their efficacy.

Interaction of Milk Production Level and Response to Diet

In several experiments, we have observed variation in individual cow response to a MFD induction diet and that high-producing cows were more susceptible to MFD risk factors. For example, Harvatine and Allen (2006) compared saturated (highly saturated prilled free FA; Energy Booster 100) and unsaturated (calcium salts of FA; Megalac R) FA supplements to a no supplemental fat control in low and high producing blocks of

cows (control 39.4 vs 47.0 kg/d, respectively). When fed the same control diet in the same barn, the low producing cows averaged 3.45% milk fat while the high producing cows averaged 3.05%. Additionally, the response to treatment differed with low producing cows having a non-significant 6% decrease in milk fat when fed the calcium salt of unsaturated FA, while the high producing cows decreased milk fat over 20%. A similar response was observed by Rico et al. (2014) when comparing a high palmitic acid supplement (87% C16:0; Berga-Fat F100) to calcium salts of palm FA (Megalac) where low producing cows numerically increased milk fat with both treatments, but high producing cows decreased milk fat and increased *trans*-10 C18:1 in milk fat when fed the unsaturated palm FA. Collectively, these studies demonstrate that there is a strong correlation between the level of milk production and diet-induced MFD. The exact mechanism is unclear, but high producing cows also have higher intakes. Increased intake is expected to increase rumen passage rate, which may modify the microbial population and increase ruminal outflow of *trans* intermediates before complete biohydrogenation has occurred. Additionally, high producing cows may differ in feeding and ruminating behavior and increased meal size or higher amount of intake after feed delivery may result in rumen acidosis.

Increasing Milk Fat Synthesis

The work around BH-induced MFD provides insight into increasing milk fat by decreasing occurrence of its inhibition. More recently, work with supplementing palmitic acid and sodium acetate has highlighted dietary methods to increase milk fat yield.

Fat Supplementation

Approximately 65% of the FA in milk are taken up by from the plasma and a large proportion of those originate from the diet. Thus, it is logical to think that increasing dietary fat would increase milk fat, but the response is very dependent on FA profile. Dietary unsaturated FA increase the risk for diet-induced MFD and commonly decrease milk fat yield. The second issue with increasing plasma FA supply to the mammary gland is that they can decrease mammary de novo FA synthesis resulting in a substitution of preformed FA for de novo FA without an increase in milk fat. Milk fat concentration and yield has been reported to be increased in individual studies by a wide array of fat supplements with differing FA profile. However, supplements enriched in palmitic acid are most consistent in increasing milk fat. This appears to be due to less inhibition of de novo synthesis by palmitic compared to other FA.

Acetate Supply

Acetate is the main VFA produced from all substrate, but is greater in fermentation of fiber than nonstructural carbohydrates (see reviews by Dijkstra, 1994, Van Soest, 1994). Acetate represents ~30% of energy absorbed by the cow and nearly 40% of milk fat originates from acetate carbon through *de novo* synthesis of FA in the mammary gland. Acetate is also an important substrate for synthesis of nicotinamide adenine dinucleotide phosphate (NADPH) required for *de novo* lipogenesis through the

isocitrate pathway, with the remaining NADPH coming from glucose metabolism through the pentose phosphate pathway (Bauman et al., 1970).

A meta-analysis based on papers published over 30 years ago (1955 to 1978) reported that acetate infusions linearly increased milk fat yield 75.5 g/d (R^2_{adj} = 0.72) and concentration 2.54 g/kg for each kg of additional acetate supplied [R^2_{adj} = 0.71; both P < 0.001, n = 24, milk yield = 14.3 kg/d; (Maxin et al., 2011)]. In more recent work, Sheperd and Combs (1998) observed a 24% increase in milk fat yield (280 g) and a 20% increase in milk fat concentration (3.41 to 4.08%) when ruminally infusing 2162 g/d of neutralized acetate for 21 d.

We first observed a 20% increase in milk fat yield (177 g/d) when ruminally infusing 424 g/d of acetate (neutralized to pH 6.1; <10% of expected acetate supply) for 4 d during an experiment investigating the effect of nutrients spared during MFD on adipose tissue (Urrutia and Harvatine, 2017). A follow-up dose titration experiment testing 0, 300, 600, and 900 g/d of ruminally infused neutralized acetate observed a quadratic increase in milk fat yield of 100, 217, and 185 g/d, respectively, compared to a sodium chloride control (equal sodium). Apparent transfer of acetate mass to milk fat was 33.4, 36.2, and 20.6% with 300, 600, and 900 g/d of acetate, respectively. In our experiments, the largest increase was in *de novo* and 16 carbon FA, but preformed FA also increased. The increase in preformed FA may indicate a stimulation of mammary metabolism or an increase in FA available to the mammary gland because of sparing of FA from oxidation in other tissues. We have also observed increased milk fat when feeding sodium acetate. For example, a seven-day sodium acetate supplementation increased milk fat 0.2 percentage units and tended to increase milk fat yield 91 g/d (Urrutia et al., 2019).

Increasing fiber digestibility and improving rumen function is expected to increase acetate supply and milk fat yield. Importantly, this is not alleviating diet-induced MFD and is separate from historical acetate deficiency mechanisms. We expect that acetate is actually upregulating lipogenesis in the mammary gland and have ongoing work to investigate the physiological effect of acetate.

Take Home Messages

- Milk fat is impacted by many dietary, genetic, and environmental factors and their interactions make it difficult to manage.
- It is important to consider non-nutritional factors such as genetic potential, season of the year, and milking sampled when setting goals and interpreting data.
- Biohydrogenation induced milk fat depression explains large decreases in milk fat and is caused by fundamental issues with stable rumen fermentation.
- Increasing dietary fat can increase milk fat, but is most consistent when feeding enriched palmitic acid supplements.
- Increasing acetate supply by increasing fiber digestibility supports higher milk fat yield.

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Managing the Diet to Control Ruminal Fatty Acid-Microbial Interactions That Reduce Milk Fat Synthesis

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Introduction

Dietary fatty acids undergo significant structural changes via a process called biohydrogenation as they pass through ruminal contents and are delivered to the intestines for absorption. A significant portion of milk fat yield, which is a primary driver of milk income, is guided by the direction of these biohydrogenation pathways. Changes in nutritional composition of the feed, brought about either by design or inadvertently because of nutritional variation in feed ingredients, can shift biohydrogenation pathways causing changes in rumen fatty acid outflow of bioactive lipids that adversely affect milk fat synthesis. Therefore, identifying the trigger that shifts fatty acid biohydrogenation in the rumen from “milkfat friendly” to “milkfat unfriendly” is of upmost importance.

The intention of this paper is to offer a possible trigger mechanism that initiates the rumen microbial population to shift its pathways of biohydrogenation toward a direction unfavorable for milk fat synthesis. Much of the direct evidence for the trigger is revealed from recent studies of isolated ruminal bacteria, in vitro rumen cultures, and cow data. Data across these studies suggests that when dietary fatty acids, coming from both the basal diet and from added fat, reaches a level sufficient to cause antibacterial effects in the rumen the result is a shift from normal biohydrogenation to an alternate pathway. The alternate pathway produces lipid bioactive intermediates that lower milk fat. The data summarized below also shows that the type and concentration of fatty acid required to reach antibacterial effects is subject to modification by other dietary nutrient considerations.

Results

What are fatty acids?

Before beginning a discussion about the fate of fatty acids as they pass through the rumen, it seems appropriate to start with a brief refresher on defining fatty acids. Put simply, fatty acids are the basic building blocks of fats just as amino acids are the building blocks of protein. Amino acids are chained together with peptide bonds in different lengths to form everything from dipeptides (2 amino acids) to polypeptides (> 10 amino acids). Fats, unlike protein, consist of no more than three fatty acids grouped together as attachments on a glycerol backbone. Fats and oils primarily consist of three fatty acids attached to glycerol referred to as triglycerides (or more correctly triacylglycerols). Forage lipids contain primarily galactolipids, where the glycerol backbone has two bound fatty acids along with a bound sugar molecule.

Fatty acids, and not the glycerol backbone, provide the benefits to animal performance, including high energy, tissue benefits, and rumen effects. For this reason,

it is important to have a basic understanding of the differences among fatty acids. Fatty acids are chains of carbons that end in an acid group, or carboxyl group as it is referred to in biochemistry. An example of a common fatty acid is stearic acid with 18 carbons and no double bonds (Figure 1). Stearic acid is low in plant oils, but present in higher amounts in animal fats, particularly in fats obtained from ruminant species such as beef tallow.

Oleic, linoleic, and linolenic acids are examples of unsaturated fatty acids containing one or more double bonds (Figure 2). Oleic acid has a single double bond between carbons 9 and 10, and is referred to as a monounsaturated fatty acid. Linoleic acid is a polyunsaturated fatty acid containing two double bonds between carbons 9 and 10, and between carbons 12 and 13. Oleic acid is the predominant fatty acid in animal fats and some plant oils, such as canola oil. Linoleic acid is the predominant fatty acid in many plant oils, including cottonseed oil, soybean oil, and corn oil. Linolenic acid, with three double bonds, is the primary fatty acid in most pasture species and in linseed oil from flax.

Figure 1. The structure of stearic acid; a saturated long-chain fatty acid.

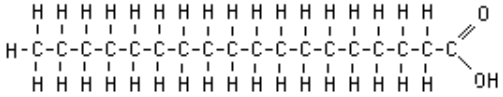
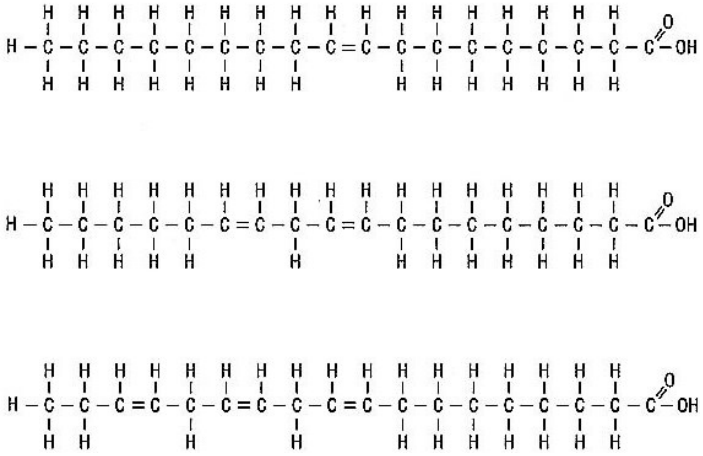


Figure 2. Structures of the three primary unsaturated fatty acids consumed by cattle, oleic acid (top), linoleic acid (middle), and linolenic acid (bottom).



Fatty Acid Input into the Rumen

Typical daily intakes of unsaturated fatty acids for diets with and without added fat are shown in Table 1. Total fatty acid concentration in feed mixes can range from less than 20 mg/g DM for basal ingredients to more than 80 mg/g DM when fat is added. Linoleic acid is the predominant unsaturated fatty acid consumed in most cases with upper intakes

exceeding 700 g/day in published studies (Table 1), or even exceeding 1000 g/day under field conditions (Chase, 2019).

Fatty acid concentration in ruminal contents reflects their concentration and variability in feed. Using results from three published studies as an example (Table 2), fatty acids varied in ruminal contents from < 10 mg/g DM for a basal diet containing 50% bermudagrass hay (Bateman and Jenkins, 1998) to 29 mg/g DM for an alfalfa/corn silage diet (Loor et al., 2002), and to just under 50 mg/g DM for diets with added plant oils. Cows grazing high quality ryegrass and clover pasture (Sun and Gibbs, 2012), interestingly, had ruminal fatty acid concentrations approaching levels observed for TMR with added fat. The implication of maintaining fatty acids in ruminal contents is that many microbial species are sensitive to high fatty acid concentrations and respond with reduced growth and metabolic activity. More specifically, antibacterial activity is greatest for unsaturated fatty acids and is not a characteristic of saturated fatty acids.

Select ruminal bacteria have an inherent protective mechanism in place designed to reduce unsaturated fatty acid concentration in the rumen and lessen the chances of antibacterial activity. This protective mechanism is referred to as biohydrogenation, where unsaturated fatty acids are enzymatically converted to saturated fatty acids (Jenkins et al., 2008). The efficiency of biohydrogenation can be seen from the results of the three studies in Table 2 where ruminal unsaturated fatty acid concentrations are much lower and less variable compared with feed or ruminal total fatty acid concentrations. Biohydrogenation, while assisting the microbial population in controlling antibacterial effects of unsaturated fatty acids, also greatly transforms the nature of fatty acid outflow from the rumen compared to its inflow from feed. This fatty acid transformation process has both positive and negative consequences on animal production and acceptability of animal based food products.

Table 1. Average intakes of major unsaturated fatty acids by dairy cattle fed a TMR without and with added fat averaged across five published studies.[1]

	DMI kg/d	Diet FA mg/g DM	18:1 g/d	18:2 g/d	18:3 g/d	RUFAL[2] g/d
Control (n=5)						
Mean	19.4	37.3	139	299	44	473
Min	12.0	18.6	53	133	26	220
Max	27.3	55.4	242	690	74	973
Fat Diets (n=21)						
Mean	19.4	59.5	280	371	56	696
Min	12.3	28.2	111	164	26	362
Max	25.7	83.5	571	710	88	1118

[1] Taken from Jenkins and Bridges (2007).
[2] RUFAL = rumen unsaturated fatty acid load (g/day) = C18:1 + C18:2 + C18:3.

Table 2. Fatty acid concentrations (mg/g DM) reported in feed and rumen samples from three studies when cows were fed a basal diet with and without added oil.

Reference/Diet	Feed	Rumen Total	Rumen Unsat.
Loor et al. (2002)			
Basal (61% alfalfa/corn silage)	35.2	28.8	**1.33**
3.5% canola oil	61.4	48.2	**2.42**
3.5% soybean oil	63.8	48.9	**1.69**
Bateman and Jenkins (1998) nonlactating cows			
Basal (50% bermudagrass hay)	14.7	8.1	**1.40**
4% soybean oil	49.7	25.0	**1.75**
8% soybean oil	83.5	32.4	**2.14**
Sun and Gibbs (2012)			
High quality pasture	42.4	46.9	**8.75**

Changes in Rumen Fatty Acid Concentration Over Time

The pathways of biohydrogenation are highly complex and yield a wide variety of intermediates. The three main unsaturated fatty acids consumed (oleic, linoleic, and linolenic acids) are all subjected to enzymatic transformations that yield a multitude of unique intermediates. As an example, the pathways and intermediates of linoleic and linolenic acid biohydrogenation to stearic acid are shown in **Figure 3** (Ferlay et al., 2017). As knowledge increases about the pathways of biohydrogenation the identity of intermediates expands. Input into the rumen of just three unsaturated fatty acids (oleic, linoleic, and linolenic acids) as raw materials leads to the production of dozens, if not hundreds, of complex fatty acid isomers in rumen outflow. Yet, this complexity of biohydrogenation is largely ignored in most discussions of biohydrogenation. Most of the attention is directed at a more simplistic version of linoleic acid biohydrogenation (**Figure 4**) that emphasizes only the few intermediates that were shown previously to inhibit fat synthesis in the mammary gland.

Very briefly, biohydrogenation of linoleic acid in the rumen begins with its conversion to conjugated linoleic acid (CLA). In this initial step, the number of double bonds remains the same but one of the double bonds is shifted to a new position by microbial enzymes. Normally, the double bonds in linoleic acid are separated by two single bonds, but in CLA, the double bonds are only separated by one single bond. Many types of CLA are produced in the rumen of dairy cows, but a common CLA produced from biohydrogenation of linoleic acid is *cis*-9, *trans*-11 C18:2.

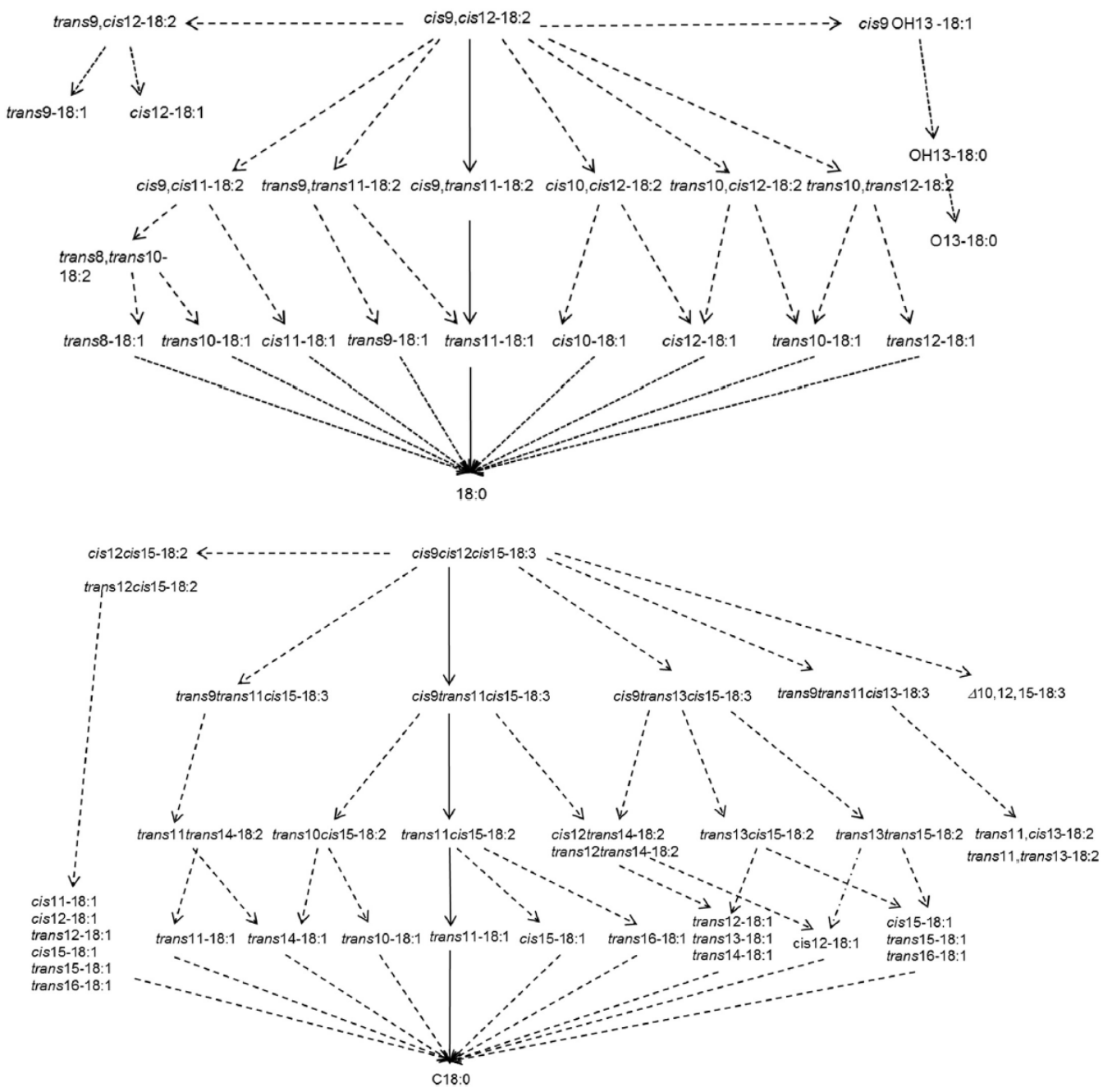


Figure 3. Proposed pathways of linoleic (top) and linolenic acid (bottom) biohydrogenation to stearic acid in ruminal contents proposed by Ferlay et al. (2017) illustrating the complexity and abundance of intermediates.

As biohydrogenation progresses, double bonds in the CLA intermediates are then hydrogenated further to *trans* fatty acids having only one double bond. A final hydrogenation step by the ruminal microbes eliminates the last double bond yielding stearic acid as the final end product. *Trans* double bonds only differ from *cis* double bonds in the placement of the hydrogens. The hydrogens are shown on opposite sides of the double bond for *trans* fatty acids, but on the same side of the double bond for *cis* fatty

acids. Although the difference in structure between *trans* and *cis* fatty acids appears small, it causes significant differences in their physical and metabolic properties. The *trans*-11 route (abbreviated as t11) of linoleic acid biohydrogenation in Figure 4 involves intermediates, including *cis*-9, *trans*-11 CLA, proven to have little effect on milk fat. The *trans*-10 route (abbreviated as t10) involves intermediates, including *trans*-10, *cis*-12 CLA, proven to reduce milk fat synthesis (Baumgard et al., 2000).

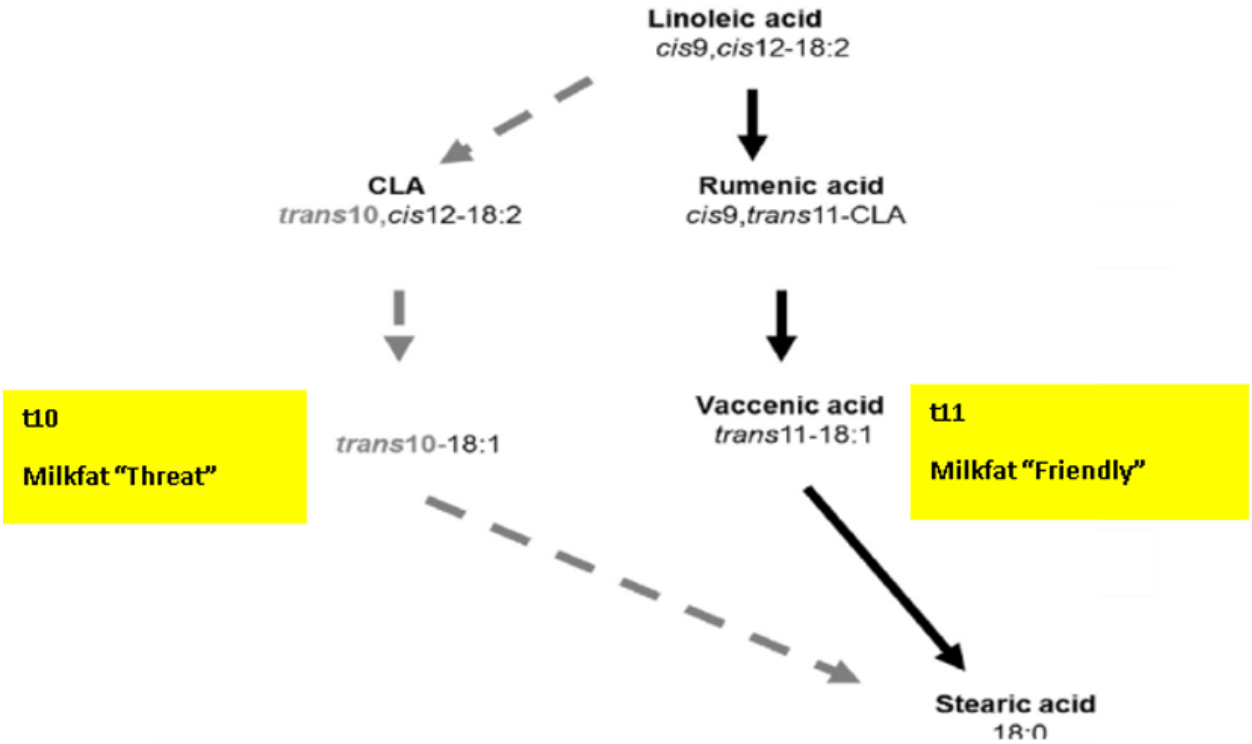


Figure 4. Simplified pathways of linoleic acid biohydrogenation emphasizing the major t11 route involving milkfat friendly intermediates and the minor t10 route involving intermediates known to inhibit milk fat synthesis by the mammary gland. Adapted from Ferlay et al. (2017).

With biohydrogenation in place, it might be argued that unsaturated fatty acid concentration remains low enough to avoid antibacterial effects. However, when the time course of biohydrogenation is examined immediately after feeding, it is common to see a large spike in unsaturated fatty acids that quickly declines over time. An example of the spike in unsaturated fatty acids immediately after feeding is shown in Figure 5. In a continuous culture study done at Clemson University, suddenly switching from a basal diet to a 3% soybean oil diet after 5 days of fermentation increased linoleic acid concentration from < 1 mg/10 ml culture contents to over 8 mg/10 culture contents by 1 hour after feeding. Sampling times earlier than 1 hour after feeding might have revealed a linoleic acid spike that was even higher. Declines in linoleic acid concentration occurred by hour 2 and then steadily declined with each advancing hour after feeding. The spike in unsaturated fatty acids immediately after feeding might induce antibacterial effects,

even though biohydrogenation maintains much lower concentrations of unsaturated fatty acids at most other times.

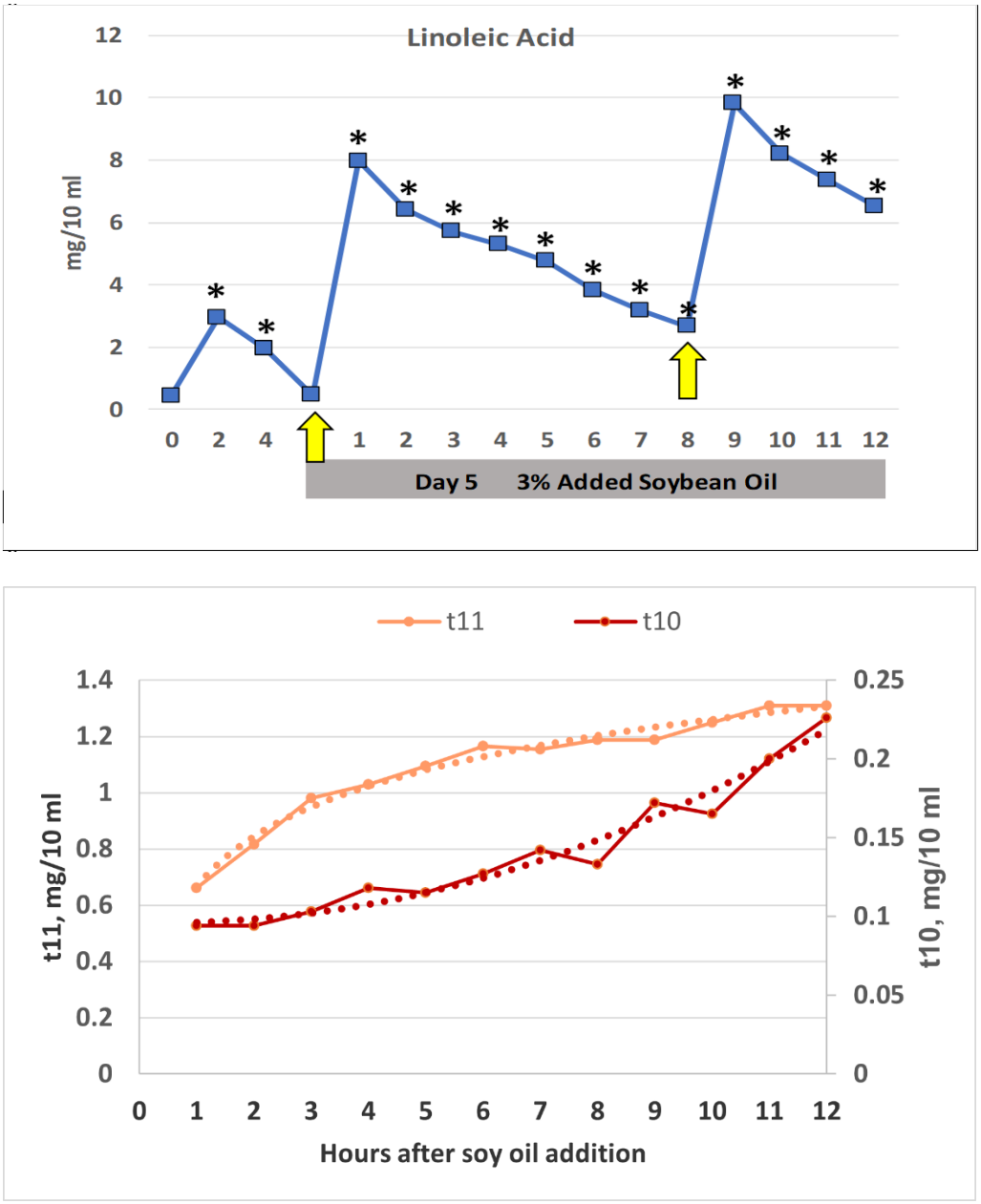


Figure 5. Concentrations of linoleic acid (mg/10 ml) and *trans* 18:1 isomers in contents taken from continuous cultures of mixed ruminal bacteria. Cultures were fed a basal diet without soybean oil for 4 days then immediately switched to a diet containing 3% soybean oil on day 5. Top graph shows linoleic acid concentrations on day 4 taken at 0, 2, and 4 hours after the morning feeding, and on day 5 taken hourly after soybean oil addition. Arrows on top graph indicate when the diet containing soybean oil was introduced into cultures. Bottom graph shows t11-18:1 and t10-18:1 concentrations taken hourly on day 5 after feeding soybean oil. Unpublished results from Clemson University.

An in vivo example of this unsaturated fatty acid spike in ruminal contents was seen in the data of Baldin et al. (2018). They reported the highest concentration of unsaturated fatty acids in ruminal contents of cows within 5 minutes of an intraruminal dose of 200 g unsaturated oil. Ruminal concentrations of unsaturated fatty acids then returned to basal values by 4 hours after dosing. This suggests that cows fed under field conditions experience a spike in ruminal unsaturated fatty acid concentration immediately after feeding that might be sufficient to cause antibacterial effects in the rumen. The amplitude of the spike would likely be a function of fatty acid input from the diet, percentage unsaturation of diet fatty acids, feeding frequency, and rate of feed consumption.

Antibacterial Effects of Unsaturated Fatty Acids

There is an extensive literature describing the antibacterial activity of various fatty acids (Desbois and Smith, 2010). Two factors that affect the antibacterial activity of lipids are fatty acid structure and concentration. Free fatty acids generally disrupt fermentation more than triglycerides and antibacterial activity of free fatty acids can be enhanced by increasing the number of double bonds (Desbois and Smith, 2010). Growth of some bacterial species is stimulated by low concentrations of fatty acids, but inhibited at higher concentrations (Maczulak et al., 1981). In attempting to predict ruminal fermentation changes caused by dietary lipid, it is often assumed that the fat load is contributed only by the fat supplement and that FFA concentration is low. Both assumptions can be wrong. Fatty acids from the TMR and forage can significantly contribute to total rumen fat load, for example when animals are consuming immature pasture. Also, FFA concentration may be elevated in some feed ingredients such as whole cottonseed stored in warm, humid conditions (Cooke et al., 2007), or in forages resulting from hydrolytic cleavage of esterified lipids during hay-making (Yang and Fujita, 1997).

The mechanism of fatty acid antibacterial effects are primarily directed at their intrusion into the bacterial cell membrane. The details of the mechanistic processes (Figure 6) can be classified based on the relationship between the following three aspects: (i) increased membrane permeability and cell lysis, (ii) disruption of electron transport chain and uncoupling oxidative phosphorylation, and (iii) inhibition of membrane enzymatic activities and nutrient uptake (Yoon et al., 2018). For anaerobes that inhabit the rumen, fatty acids exert antibacterial properties through disorganization of the cytoplasmic outer membrane that can lead to increased membrane permeability and even cell lysis, inhibition of membrane enzymatic activity, and impaired nutrient uptake.

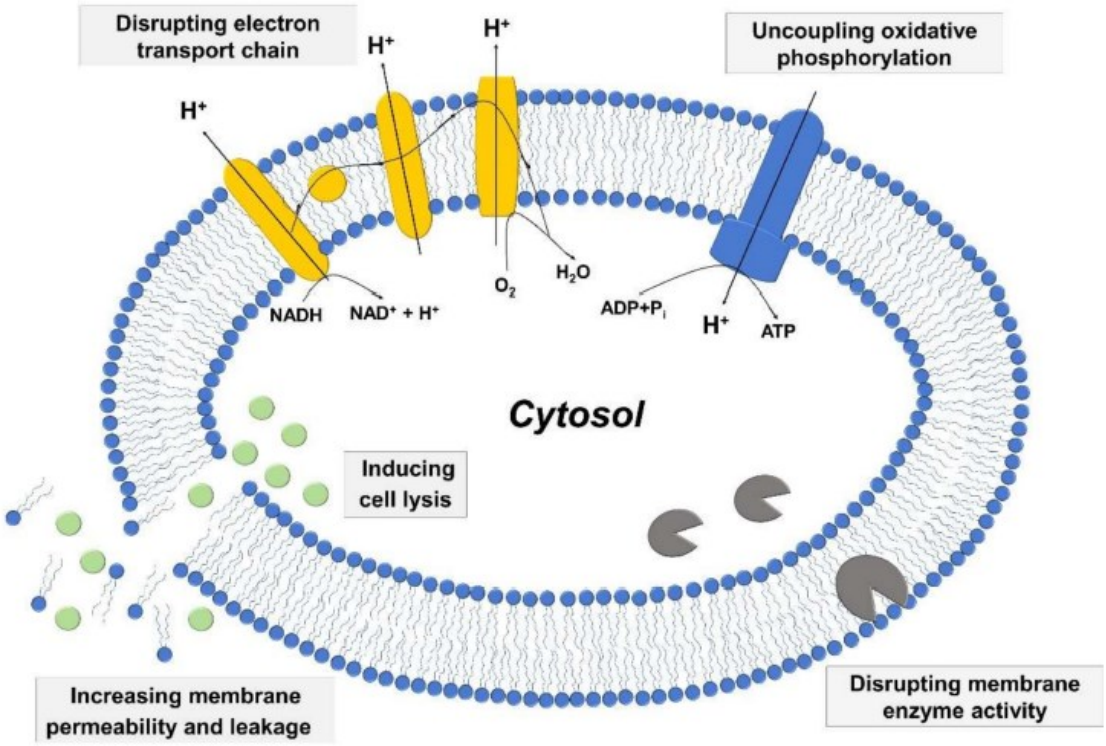


Figure 6. Mechanisms of antibacterial activity of fatty acids (Yoon et al., 2018). For ruminal microorganisms, fatty acids exert antibacterial properties through disorganization of the cytoplasmic outer membrane that can lead to increased membrane permeability and even cell lysis, inhibition of membrane enzymatic activity, and impaired nutrient uptake.

Several properties of antibacterial effects in the rumen directly impact the t10 versus t11 pathways of biohydrogenation and the eventual impact on milk fat.

1. One important factor is that fatty acids appear to exhibit antibacterial effects quickly. Maia et al. (2010) reported a > 96% reduction in metabolic activity in the ruminal bacterium *B. fibrisolvens* within 20 minutes following the addition of 0.2 mg/ml of linoleic acid to cultures. A recent continuous culture trial done at Clemson University examined how quickly soybean oil shifted biohydrogenation pathways from the normally predominant t11 pathway to the minor t10 pathway. Cultures were maintained on a basal diet for 4 days with t10-18:1 and t11-18:1intermediates analyzed just before the morning feeding (8 am) and then again at 2 and 4 hours after the morning feeding. On day 5 the cultures were suddenly switched to a diet containing 3% added soybean oil with samples analyzed every hour for 12 hours. The results (Figure 5) revealed an escalation of the t10 pathway within a few hours after introducing soybean oil into the cultures. This could mean that unsaturated fatty acid concentration may not need to be sustained at high levels at all times to cause antibacterial effects. Perhaps just the transient peak in ruminal unsaturated

fatty acid concentration that occurs immediately after feeding is sufficient to trigger antibacterial effects.

2. A second critical point of antibacterial activity in the rumen is that not all bacterial species are equally susceptible. Disruption of membrane integrity following the addition of linoleic acid to cultures ranged widely across 17 species of ruminal bacteria (Maia et al., 2007) monitored by fluorescence techniques. Generally the bacterial species following the t11 route of biohydrogenation showed greater disruption of membrane function, including > 50% disruption for *Butyrivibrio spp.* and > 90% disruption for *Pseudobutyrivibrio*. Membranes of *M. elsdenii* that follows the t10 route of linoleic acid biohydrogenation were < 5% disrupted by the same dosage of linoleic acid. Thus, fatty acid concentrations above the antibacterial threshold cause selective damage in the rumen depending on bacterial species, with less damage seen for t10 microorganisms.

3. Third, not all unsaturated fatty acids have equal propensity to cause antibacterial effects at the same concentration. For instance, relative inhibitory effects of individual fatty acids on growth of *B. fibrisolvens* was linolenic>linoleic>oleic>stearic according to Maia et al. (2010). The general trend was greater inhibition with increasing number of double bonds in the acyl chain. Similar trends have been reported in vivo. Dorea and Armentano (2017) reported feeding saturated fatty acids to cows, such as palmitic acid, increased total milk fatty acids, mainly by increasing milk C16 yield. However, feeding unsaturated fatty acids decreased total milk fatty acid by inhibiting secretion of milk fatty acids shorter than C18, with linoleic acid being more inhibitory than oleic.

4. A fourth and perhaps most significant property of antibacterial effects is that the threshold to cause a shift in the pathway of biohydrogenation is modified by environmental and chemical conditions in the rumen. If the threshold was a constant concentration of unsaturated fatty acids in ruminal contents then feeding recommendations for fat could be modelled much easier. Instead, low pH and lactic acid accumulation were both shown to accentuate antibacterial effects of unsaturated fatty acids, specifically targeting t11 microorganisms (Maia, personal communication and Maia et al., 2010). Both of these conditions implicate high starch levels as a negative predictor of milk fat synthesis.

Conclusions

Using the antibacterial switch described in this paper, a sequence of events can be suggested whereby the pathways of biohydrogenation change course moving the rumen from milkfat “friendly” to milkfat “unfriendly”. The initial step is for unsaturated fatty acid concentration in the rumen to exceed the threshold for antibacterial effects. This could happen in one of two ways; 1) increase dietary concentration of unsaturated fatty acids that could arise from variation in basal fatty acids or from added fat to the diet, or 2) lower the antimicrobial threshold. The threshold that ruminal microorganisms can tolerate is lowered by increasing starch, lowering rumen pH, increasing lactate, or a combination of

these. Once the antibacterial threshold is reached, t11 microorganisms respond within hours by shutting down metabolic activity including rates of biohydrogenation. This reduces the flux of linoleic acid flow through the normal t11 pathway of biohydrogenation. Consequently, because t10 microorganisms are less sensitive to antibacterial effects, more linoleic acid is now available for biohydrogenation through the alternate t10 pathway. With each subsequent feeding of the same diet the accumulation of CLA (specifically *trans*-10, *cis*-12) in the rumen continues providing a steady CLA flow to the mammary gland where *de novo* fatty acid synthesis is inhibited.

Some high producing herds consume in excess of 1000 g unsaturated fatty acids per day but still maintain milk fat around 4% (Chase, L. E., 2019). Other herds experience milk fat depression with <500 g of unsaturated fatty acids. McCarthy et al. (2018) failed to detect a significant relationship between milk fat yield and intake of unsaturated fatty acids across 79 herds in the northeast and upper Midwest. This variation clearly shows that unsaturated fatty acid intake alone is not a good predictor of milk fat. Models predicting milk fat should include all factors known to affect their antibacterial effects including amount and type of starch, rumen pH (effective fiber, type and amount of buffers, TMR mixing, etc), and fatty acid release rates from plant structure.

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Use of an On-farm Application to Maximize Milk Fat Production: What We Have Learned

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Elanco Animal Health

In virtually every dairy market, milk fat yield is an important component of milk pricing. While different regions in the U.S. have different pricing formulas, all of them use milk fat (or "butterfat") yield as part of the price calculation. In addition, over the last several years, 2015 to 2019 in particular, the value of milk fat relative to other components of milk, whether protein or skim, has significantly increased. It has only been in the last six months or so that the value of milk protein has recovered to a significant degree relative to milk fat (USDA-Economics, Statistic and Market Information System).

Milk fat content or more commonly, "milk fat percent", is the traditional – and, therefore, in some ways, easiest to communicate – metric associated with milk fat production. Having a goal for milk fat percentage is reasonable, but it should also be noted that dairy producers are paid for milk fat by *weight*, not *concentration*. This is important since it is possible to have no change, or even a slight reduction, in milk fat percent, yet produce more *pounds* of milk fat overall, if overall milk production increases. Nevertheless, milk fat percent, even with these obvious drawbacks, is the most common way that producers monitor milk fat performance.

An obvious question then is "what is an acceptable milk fat percent for a given farm?". This answer varies according to the goals and expectations of the dairy management team. It is worth noting that milk fat concentration in U.S. Holstein cows in the U.S. has increased over the last several years(Elanco Animal Health), and based on current data, a milk fat percentage of 3.8 or more in all Holstein herds is very attainable while also achieving high production.

How much can we influence milk fat performance? Milk fat is highly variable – both within a herd and across herds – suggesting that there could be many identifiable factors which could account for this variation, and indeed a great deal has been discovered about the variation in milk fat production in recent years. There are many known nutritional factors that can influence milk fat production, but there are also many non-nutritional factors that should be considered in an investigation of milk fat performance. For example, herd lactational and genetic demographics, feeding management practices, and housing systems can each play an important role in this multifactorial outcome. Like nutritional factors, most of these non-dietary factors can be managed. So, milk fat performance is influenceable, and it can be affected in meaningful ways.

One way to approach the issue of milk fat production is to think of it systematically in terms of three broad areas: herd demographic factors, nutritional factors, and management factors. There are many specific factors within these three categories, but it is helpful to start with a well-defined, repeatable approach. *Herd demographics* include

factors like stage of lactation dynamics, parity distribution, and genetic potential. *Dietary factors* are the specific concentrations of various critical nutrients that the cows consume. *Management factors* represent the decisions that management has made which can influence accurate delivery of the diet or feed access by the cows.

Herd Demographics

Maybe the most profound example, and one we do not have as much control over, is seasonality. In North America season of the year has a significant biological effect on milk fat percentage – specifically, milk fat yield decreases in the summer months. This decrease can be exacerbated by heat stress but is more biologically fundamental than simply a heat stress effect. For example, milk fat concentration begins to decrease in February and March – much earlier than would be expected if the effect were simply a consequence of heat stress. The yearly change in photoperiod with lengthening and shortening days likely plays a role in seasonal milk fat performance (Salfer et al. 2019). It is important to keep this in mind when setting goals or expectations for milk fat yield in the summer.

Other slightly more controllable demographic factors include genetic potential, which could be evaluated by Predicted Transmitting Ability or genomic testing, parity distribution – the proportion of milking cows that are in each lactation, and the distribution of Days in Milk (DIM).

Dietary Factors

While there are many nutritional factors that could limit milk fat, most of them have their effect by:

1) altering the rumen environment, and/or
2) affecting the amount of unsaturated fatty acid in the rumen.

Since unsaturated fatty acids must be converted to saturated fatty acids in the rumen in a process known as *biohydrogenation*, anything that interferes with or overloads this critical process can limit milk fat synthesis. For example, too much starch or too little digestible fiber in the diet can lead to extremes in rumen pH, which may hinder biohydrogenation. Simply overfeeding unsaturated fatty acids can overwhelm the system and allow potent inhibitors of milk fat synthesis to escape the rumen. These are key examples of how the diet itself can affect milk fat concentration and yield (Bauman and Griinari, 2003).

Other dietary considerations include dynamics of dry matter intake, the feed delivery system, the number and timing of feedings, the moisture content of the diet, fiber and fiber digestibility, starch and starch digestibility, fat (and especially unsaturated fat) content of the diet, buffer feeding, dietary cation-anion balance, and amino acid balance.

Management Factors

Many of the management factors have to do with feed access. If you consider, for example, three areas of management: stocking density, time required for milking, and empty feed bunk time, these seem like independent concerns of management. In reality, they all affect the amount of time a cow can spend at the feed bunk eating and other aspects of feeding behavior. So even the perfect diet on paper cannot perform as intended in the cows if management conditions and management decisions do not enable great nutritional performance.

Other controllable factors in this category of management factors include mixing uniformity, timing of feed delivery, frequency of feed delivery, ingredient load order into the feed mixer, proper functioning of the feed mixer, frequency of feed "push-up", feed stability in the bunk, wet forage storage system and frequency of moisture testing and associated ration adjustments, heat stress abatement, stocking density, time away from the home pen for milking, level of feed sorting, duration of empty bunk time, water quality and potential water contaminants or dissolved minerals, and yeasts and mycotoxins.

A systematic "milk fat assessment". An application (Milkfat dTect™) has been developed by Elanco Animal Health which incorporates farm-specific inputs in each of these broad categories (Herd Demographics, Dietary Factors, and Management Factors; Figures 1, 2, and 3).

This digital tool, developed for the iPad®, enables a thorough process of on-farm observations and consultation with the nutritionist to assess each of these areas in great detail. Milkfat dTect includes user aids to assist in key calculations, and photos and notes can be added during the assessment. The application then generates a report which organizes the findings into these three categories and applies a risk score to the various findings within each area (Figure 4). The findings are supplemented with literature citations to support their importance in milk fat performance. This allows the nutritionist to focus on areas of concern and prioritize forthcoming action with the producer to improve milk fat production. The summary report also includes photos and notes captured during the assessment. Milkfat dTect can be used as a troubleshooting tool for milk fat depression or to identify the constraints which may prevent maximizing milk fat yield.

An additional benefit of using a tool like Milkfat dTect is the ability to permanently store and organize the data. Data collected during assessments is automatically saved in a secure, remote database for access later. This creates a unique opportunity to use the data to monitor performance within a herd, create summaries and make comparisons across herds, and collect data for field-based research. All the key data items are saved in a structured environment for more advanced analytical investigation (Figure 5).

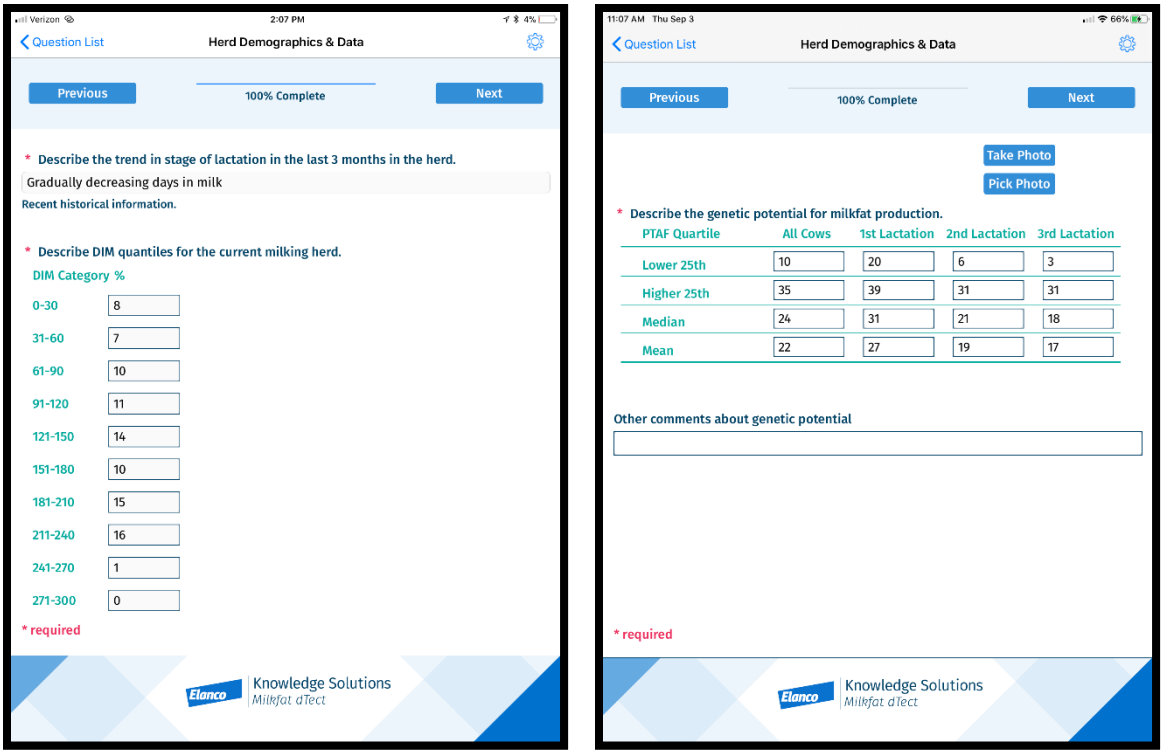

Figure 1. Example “Herd Demographic” Data

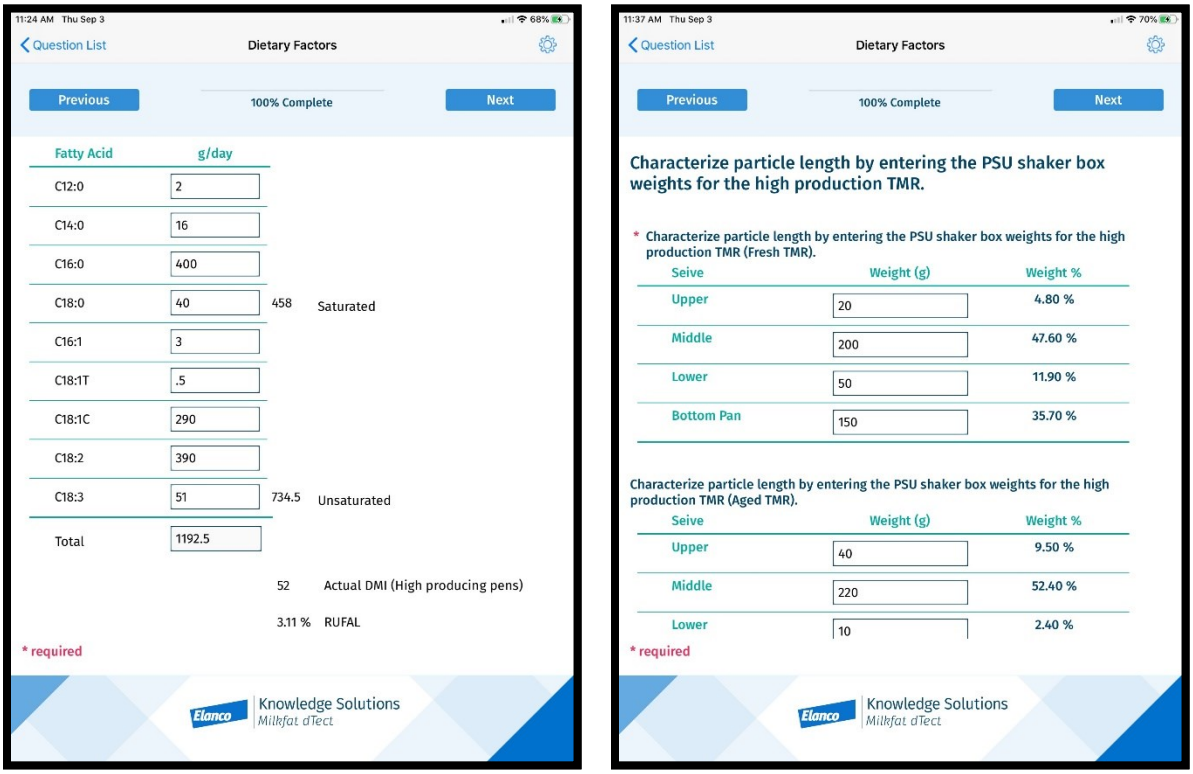

Figure 2. Example “Dietary Factors”

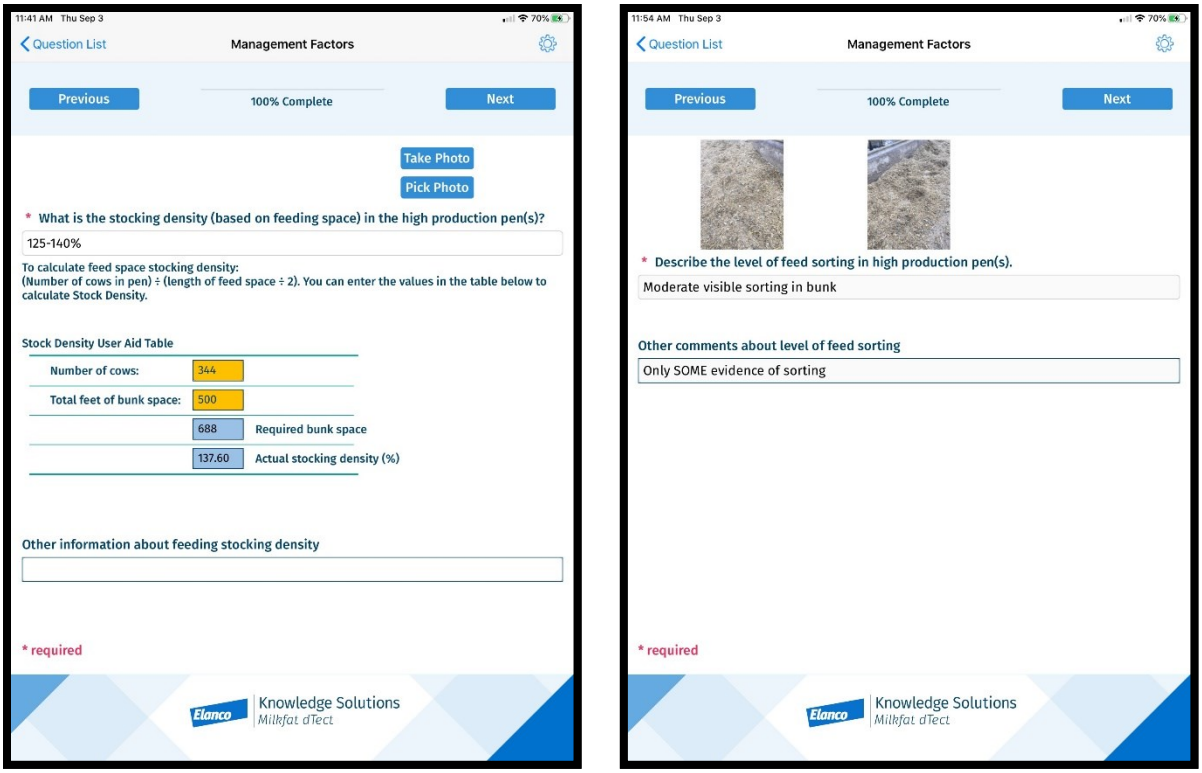

Figure 3. Example “Management Factors”

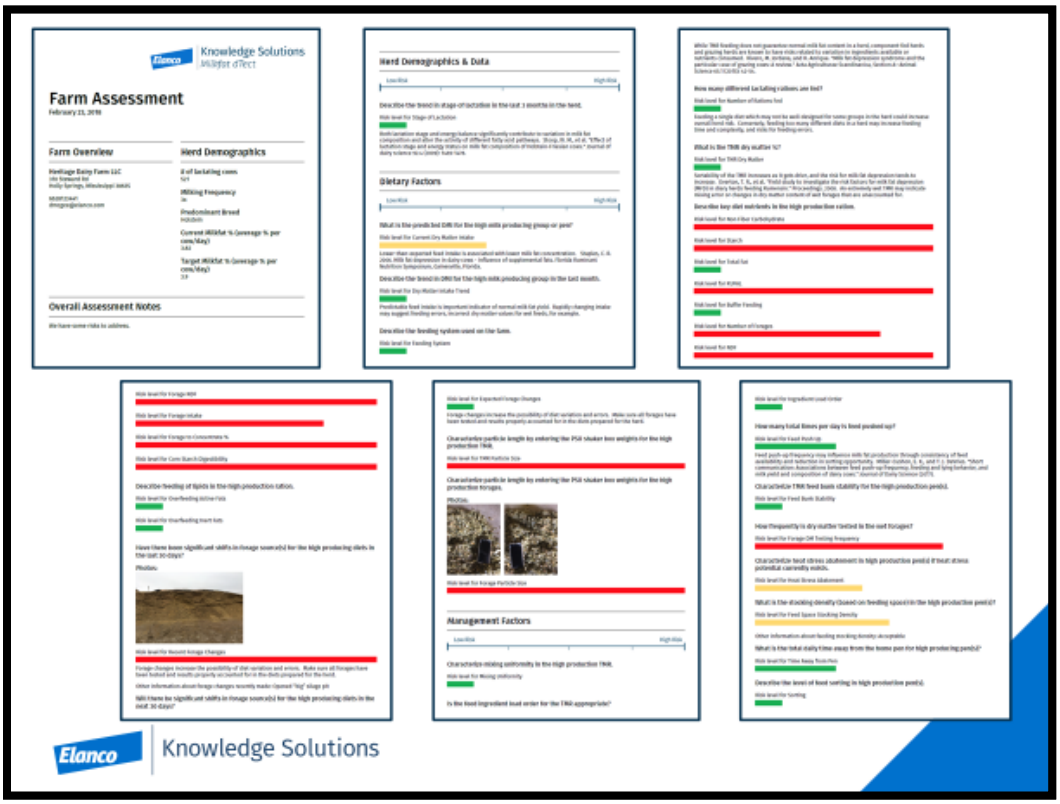

Figure 4. Example Milkfat dTect Report

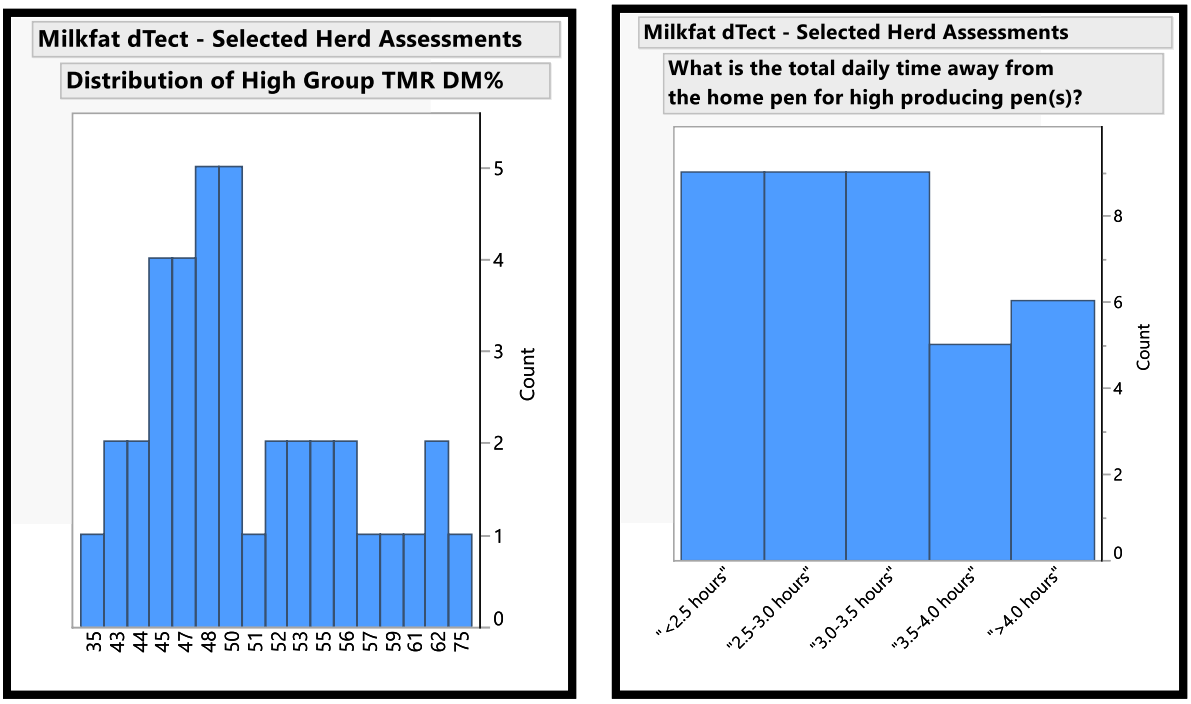

Figure 5. Example "Multiherd Comparisons"

Key Take Aways

- Milk fat is valuable and maximizing milk fat yield, not just concentration, is crucial to the financial success of a dairy operation.
- Maximizing milk fat is a multifactorial process and requires a multifaceted approach to achieve the goal.
- Factors involved in milk fat yield can be grouped into three categories: Demographic factors, Dietary factors, and Management factors.
- Elanco can help provide a very thorough, structured approach to the challenge of maximizing milk fat yield by working with dairy nutritionists and their clients with Milkfat dTect™
- Advanced multiherd analyses and comparisons are enabled through maintenance of assessment data in a secure, accessible, remote database.

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Our Food Industry Today: Issues and Opportunities

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Introduction

During the COVID-19 pandemic, the essentiality of food for human life and societal functioning has come into sharper focus, especially for citizens of wealthy nations who may have not experienced significant food insecurity for a generation or more. A common sight in US retailers in the spring of 2020 was empty cases of meat, milk, and eggs due to large and fast shifts in the point-of-purchase (more retail, fewer food service meals) and disruptions to supply chains and processing due to COVID-19 outbreaks (e.g., meat processing plant closures).

Despite these disruptive impacts of coronavirus, the recent trends in the food industry of focusing on environmental sustainability and animal welfare issues remain. Alternatives to animal source foods, such as plant-based meats and milks, continue to garner attention and are often marketed as healthier, more sustainable, and animal-friendly food items. This proceedings paper will provide a primer of the key issues that the food industry and animal agriculture are facing in the broader topic area of environmental sustainability, highlight some of the major commitments that have been made by companies and industry groups as they relate to animal agriculture, and discuss the opportunities and challenges for animal agriculture in sustainability.

Key Issues Related to Sustainability and Animal Agriculture

Sustainability cuts across environmental, social, and economic domains, or as some refer to in the business community, the “triple-bottom line” (Elkington, 1994). The 1987 report “Our Common Future” offered a widely cited definition of sustainable development that is often applied to sustainability more generally: meeting “the needs of the present without compromising the ability of future generations to meet their own needs” (Bruntland, 1987). While there is wide agreement that sustainability must balance environmental, social, and economic concerns and take a long-term focus, there are different views as to what this means for animal agriculture. The divergent viewpoints are caused by different formulations as to what the most pressing problem(s) or challenges are in agriculture. This can be illustrated by the different “schools of thought” put forward by Beede (2013):

- Food security (“Pro-Production”)
- Environmental stewardship (Pro-Environment)
- Society (Pro-Society)

None of these schools of thought are mutually exclusive, but they do help explain why arguments regarding sustainability can seem intractable. To an individual that places the highest priority on food security, any change to a production system in the name of sustainability that may decrease overall production may be viewed as no solution at all. To an individual with an environmental stewardship/pro-environment focus, an agricultural system that produces ever more food output while degrading underlying soil and water resources may be seen as fundamentally flawed. To an individual with a pro-society focus, a system that does not place people as central to sustainable production (e.g., livelihoods of farmers and communities) is missing the bigger holistic picture. Acknowledging these different schools of thought is important to have meaningful discussions. In the United States, the food security/pro-production view is often dominant in the commercial agricultural sphere as evidenced by a focus on "feeding the world" in messaging and an emphasis on increasing productivity.

In summary, sustainability is a complex issue filled with subjective value-judgements. Questions about sustainable animal-source food production are questions about what the future **should** look like.

Climate Change

While sustainability is a wide-ranging topic area, climate change has dominated the sustainability discussion as it relates to food, with measures such as carbon footprints (i.e., greenhouse gas emissions per lb. of food produced) often standing in as proxies for sustainability. Since the Industrial Revolution began, global average temperatures have increased in part due to human activity increasing the concentrations of greenhouse gases in the atmosphere. Animal agriculture both contributes to the issue of climate change through the emission of greenhouse gases and is impacted by climate change, through issues such as heat stress, potential increased disease incidence, and potentially negative impacts on feed availability and quality (IPCC, 2013).

The major greenhouse gases of importance from animal agriculture are carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). The gases have different potentials to trap heat in the earth's atmosphere and different atmosphere half-lives. To compare across gases, global warming potentials are often used to put all gases on a carbon dioxide equivalent (CO_2e) basis. The latest 100-year global warming potentials of CO_2, CH_4, and N_2O are 1, 28, and 265, respectively (IPCC, 2013). Newer research has suggested this system of CO_2e masks the impacts of either increasing or decreasing rates of emissions of short-lived climate pollutants such as CH_4. A system of carbon dioxide warming equivalents (CO_2we) has been proposed as an alternative to better represent the connection of CH_4 emissions to impacts on global temperature change (Cain et al., 2019a). Such a new system is of importance to animal agriculture as methane emissions are dominant, in particular for ruminant animal systems as enteric methane represents 46.5% of cattle milk and 42.6% of cattle meat greenhouse gas emissions globally from a life cycle perspective (Gerber et al., 2013). Under the system of CO_2we, a 0.3% reduction in CH_4 emissions per year, equates to zero temperature change impacts (Cain et al., 2019b).

Soil

Soil is critical for nearly all the food humans consume. Soil loss has been significant throughout agricultural history and in the United States, cropland soils still lose an average of 4.62 tons per acre per year (1.91 from wind and 2.71 from water erosion; U.S. Department of Agriculture, 2018). Uncultivated soils, such as pasture and rangelands, tend to lose far less soil per year, as continuous cover of the soil and roots help hold the soil in place when faced with water and wind pressures (Table 1).

Table 1. Estimated average annual sheet and rill erosion (water erosion) on non-Federal rural land in New York by year (tons per acre per year; U.S. Department of Agriculture, 2018).

Year	Cultivated cropland	Non-cultivated cropland	Total cropland	Pastureland
2002	2.96	0.72	1.75	0.28
2007	2.94	0.74	1.79	0.42
2012	3.30	0.81	2.11	0.45
2015	3.41	0.77	2.17	0.44

Soil health has been a topic of increasing interest in agriculture and the food industry. Soil health can be defined as the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans (USDA-NRCS). Improving the health of soils can have wide-ranging benefits, such as boosting crop yields, enhancing water quality, increasing resilience to drought, reducing greenhouse gas emissions, increasing soil carbon sequestration, increasing the provisioning of pollinator habitat, and building disease suppression (Soil Health Institute, 2020).

Water

Water quality and quantity are pressing issues for animal agriculture; however, they tend to be much more localized issues as compared to climate change and greenhouse gas emissions. Within the United States, there's considerable geographic variation with regard to water availability and water stresses. For animal agriculture, most of the water use is actually embedded in feed, meaning the water required to grow animal feeds. For example, 98% of the water use associated with U.S. beef production from cradle-to-grave is used to grow feed, while drinking water represents less than 1% of water use (Asem-Hiablie et al., 2019).

Surface and groundwater quality can be impacted by animal agriculture through nutrient runoff and leaching from manure applied to soils, manure left on pastures, from manure storage and animal housing, or from synthetic fertilizers used in the growing of feed for farm animals. Particular nutrients of concern are nitrogen (N) and phosphorus (P), both of which are often limiting in natural ecosystems. When N and P from animal agriculture sources reach surface waters, they may cause eutrophication. Nitrate is a pollutant of concern for groundwater, as it can cause human health impacts (e.g., blue-baby syndrome). As livestock operations have increased animal units per farm and

concentrated in geographic locations within the U.S. and other developed nations, concerns about nutrient loading and mismatches between N and P mass balances have grown (Knowlton and Ray, 2013).

Air Emissions

Besides greenhouse gas emissions, animal agriculture can be a source of other air quality pollutants, namely emissions of reactive nitrogen species like ammonia (NH_3) gas. Ammonia emissions from livestock production represent approximately 60% of total global NH_3 emissions (Uwizeye et al., 2020). Approximately, 25 to 50% of the feed N consumed by beef and dairy cattle can be emitted as ammonia gas (Hristov et al., 2011). Ammonia can lead to the formation of particulate matter less than 2.5 microns in diameter, which can negatively impact human health and visibility, and NH_3 can contribute to eutrophication of water when deposited, and the acidification of ecosystems (Hristov et al., 2011).

Animal agriculture can also lead to emissions of dust from outdoor lot housing systems and volatile organic compounds (VOCs). Emissions of VOCs are important precursors to the formation of troposphere ozone, which is a health concern for human, plant, and animal life, as well as an important constituent in the formation of smog. Some VOC emissions can come from animal manure, but research has demonstrated that fermented feeds (i.e., silages) tend to be a more significant sources (Place and Mitloehner, 2013).

Resource Competition:

Animal feed-human food competition is an issue that has often been raised with regard to sustainable animal agriculture. There are concerns that animal feed can directly compete for human food and/or that animal feed is grown on lands that would produce more food if used to grow food crops instead of animal feed.

Feed-food competition varies by species and production system, but in general, ruminant agriculture systems tend to have lower direct feed competition as compared to monogastric agriculture systems. This is due to the differences in digestive anatomy and the higher proportion of so-called industrialized systems for global pork and poultry production. Grains make up 13% of the global livestock (monogastrics and ruminants combined) feed ration; however, grains only represent 4.3% of the global ruminant ration (Mottet et al., 2017, 2018). Often, the protein quality of feed inputs used by livestock are of lower quality, hence livestock, can upcycle low quality and inedible protein sources into nutrient-rich meat, milk, and egg proteins.

When considering arable land use, a higher proportion of monogastric feed (essentially all) comes from arable lands, as compared to approximately 14% of total ruminant land use (Table 2). Improvements of lifetime feed conversion efficiency though improved genetics, nutrition and feed digestibility, and better husbandry and health

outcomes can reduce feed-food competition both from a standpoint of direct feed use competition and land use (Mottet et al., 2017).

Table 2. Global estimates of ruminant and non-ruminant protein production and land use for developed (OECD) and developing (Non-OECD) countries. Adapted from Mottet et al., 2017.

	Non-OECD		OECD		World		
	Ruminant	Monogastric	Ruminant	Monogastric	Ruminant	Monogastric	Total
Protein production, million metric tons/yr	22,095	25,576	14,260	12,670	36,355	38,246	74,601
Grasslands suitable for crops, ha[1]	576.1	0	108.8	0	685	0	684.9
Grasslands unsuitable for crops, ha	1212.2	0	52.2	0	1260.4	0	1,260.4
Cereal and legume silage, fodder beets, ha	55.9	0	10	0	65.9	0	65.9
Cereal grains, ha	43.4	98.5	28.9	39.7	72.3	138.2	210.5
Oil seed and oil seed cakes, ha	23.6	70.4	8.4	28.9	32	99.3	131.3
Other crops, ha	0	2.1	0	0.8	0	2.9	2.9
By-products, ha	24.2	4.2	4.2	0.5	28.4	4.7	33.1
Crop residues, ha	118.5	4.2	3.1	0.3	121.6	4.4	126.0
Total arable (incl. silage/fodder, other, byproducts, crop residues), ha	265.6	179.4	54.6	70.2	320.2	249.5	569.7
Total grasslands, ha	1788.3	0	161	0	1945.3	0	1,945.3
Total land area, ha	2053.9	179.4	215.6	70.2	2266	249.5	2,515.0

[1]While the authors have classified these hectares as suitable for crops, many of these grasslands are located in areas where conservation organization groups are attempting to prevent further conversion to croplands to preserve wildlife habitat, maintain soil carbon stores, and protect water quality (e.g., Northern Great Plains region; WWF, 2020)

Food Company and Industry Commitments

The increasing societal interest in environmental sustainability, climate change, along with animal welfare and antimicrobial resistance has led some corporations and industry associations with ties to animal agriculture to develop commitments and other strategies to demonstrate that they are good societal actors. Investors are increasingly interested in these topics as evidenced by this year's letter-to-CEOs by Larry Fink, the CEO of Blackrock, an investment fund with $7.4 trillion of assets under management. From Mr. Fink's letter, "*Our investment conviction is that sustainability- and climate-integrated portfolios can provide better risk-adjusted returns to investors. And with the impact of sustainability on investment returns increasing, we believe that sustainable investing is the strongest foundation for client portfolios going forward*" (Fink, 2020).

A popular strategy for food and agriculture companies, whether public or private, is to work with the Science-Based Target Initiative (SBTI) to set goals for their company's greenhouse gas emissions to stay within certain global temperature targets (e.g., 1.5˚C global average temperature change from pre-Industrial times). The SBTI is a collaboration between the Carbon Disclosure Project (CDP), the United Nations Global Compact

(UNGC), the World Resources Institute (WRI), and the World Wildlife Fund of Nature (WWF). Greenhouse gas emissions considered by companies typically include at least Scope 1 (the company's own operations) and Scope 2 (electricity/energy use emissions) emissions but increasing include targets for Scope 3 emissions (supply chain emissions, company travel, etc.) as well. For animal agriculturalists, emissions from their own operations (the farm's Scope 1 and 2 emissions) is now often accounted for in food companies' Scope 3 emissions goals or targets. Food and beverage companies that have animal-sourced food production within their supply chains that have set SBT include, Danone, Mars, Nestlé, Tyson Foods, Cargill, Coca-Cola, Stonyfield, and Maple Leaf Foods (SBTI, 2020).

Additionally, the cooperative/dairy processor Dairy Farmers of America recently set their own SBT for a 30% reduction in their direct and supply chain greenhouse gas emissions by 2030 relative to the year 2018 (DFA, 2020). The U.S. dairy industry has set a Net Zero Initiative goal, meaning, net zero greenhouse gas emissions (sources + sinks) by 2050 along with goals to optimize water use and improve water quality (Hershey, 2020). Outside of the U.S., the Australian red meat organization, Meat and Livestock Australia (MLA), has set a goal of becoming carbon neutral (sources + sinks of greenhouse gases) by the year 2030 (MLA, 2020). Work is ongoing at the U.S. Roundtable for Sustainable Beef to develop goals for the organizations "high priority indicators" of sustainable beef production, which includes air & greenhouse gas emissions with the target for the goals becoming public in 2021. In summary, both food companies, processors, and producer-associations are setting goals as they relate to sustainability, with an emphasis on greenhouse gas emissions.

Opportunities and Challenges

The food industry and animal agriculture are in a time of rapid changes with regard to sustainability. Increased public attention and public commitments are drawing a spotlight on the industry, thereby giving animal agriculturalists an opportunity to demonstrate their commitment to being good community members, stewards of the land and water, caretakers of animals, and innovative food producers. This spotlight is an opportunity for animal agriculture to counteract the narrative that animal agriculture cannot improve its sustainability enough to meet future food demand, which is a strong motivating factor behind calls to shift diets away from meat, milk, and eggs and the adoption of more alternative or imitation proteins.

However, there will be challenges to meeting sustainability goals. These challenges can be bucketed in three large categories: 1. new innovations and technologies (development and acceptance), 2. adoption and producer economics of new innovations and technologies, and 3. measurement or verification of sustainability outcomes.

New innovations and technologies can encompass a wide range of practices and technologies from anaerobic digesters, to wearable devices for cows, to feed additives that reduce enteric methane, to genomic selection indices that incorporate greenhouse

gas emissions. While there are examples of deployed or nearly deployed technologies in all these areas, further innovations do require significant research and development funding either from private companies, public sources, or public-private partnerships. In the past few decades, public research investment in animal agriculture has been stagnant to declining, which presents a challenge: more societal demands are being put on animal agriculture and yet societal funding of research and development in animal agriculture is not significantly increasing. Additionally, there can be challenges with consumer perception of new technology solutions, as evidenced by the near complete removal of the technology recombinant bovine somatotropin that did have a demonstrated ability to lower the greenhouse gas emission intensity of milk production. As mentioned earlier in this paper, value judgements are an important part of sustainability and ignoring social concerns, whether real or perceived, over a technology's impact on food safety or animal welfare can mean societal rejection of the technology (National Research Council, 2015).

Any new technologies or innovations must be adopted on commercial farms to have an effect. Limitations to adoption can be related to technical understanding, but also the capital investment requirements or potential increase in operating expenses. For example, some feed additives in development have shown promise to reduce enteric methane emissions in controlled research environments. However, the adoption of these feed additives will depend on if the additive is able to generate returns above costs, whether through improvements in efficiency (in some cases, reducing enteric methane may increase the feed efficiency of ruminant production), or through compensation schemes from the processors or purchasers of animal products, or through public programs. Given the economic challenges faced by many in animal agriculture, it should be viewed as a non-sustainable "sustainability solution" to increase producers costs to mitigate greenhouse gas emissions without compensation.

Finally, covering costs and potential compensation highlights the last main challenge: on-farm measurement and verification. Currently, there are tools that can be used to estimate greenhouse gas emissions, such as the Cool Farm Tool; however, often these tools are limited in how they can capture the impacts of new technologies, interventions, or innovations. Continuous updating of such tools, or development of new tools that can interface with herd management software for example, may be required to better verify changes in estimated greenhouse gas emissions and other nutrient losses or resources used. On-farm direct measurement of greenhouse gas emissions can be costly and infeasible, thus individual or combinations of proxies for greenhouse gas emissions (e.g., dry matter intake, feed composition, and milk fatty acid profile to predict enteric methane emissions) may need to be validated and deployed to facilitate verification of goals such as U.S. dairy's Net Zero Initiative.

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Nutraceuticals as an Alternative Strategy for the Use of Antimicrobials

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Introduction

A more extensive review of literature on the topic is available at Ballou et al. (2019) Vet. Clin. North Am. Food Animal Pract. 35:507-534. Zoonotic multi-drug resistant bacterial strains and antibiotic resistance has encouraged changes to on-farm use of antimicrobial use. Veterinarians and producers are evaluating alternatives to the use of antimicrobials. Nutraceuticals, primarily derived from microbial and plant-based compounds, are receiving increased research and commercial attention. Nutraceuticals are a diverse group of compounds and microbes that offer some advantageous effects to health and productivity, including improved feed efficiency or reduced disease through either immune modulation or decreased infection.

Nutraceuticals can be classified in many different ways and the more common are based on the mechanism of action, chemical structure, or the source of the compounds. In this proceeding the focus will be predominately through the mechanism of action. We will discuss 3 broad classes of nutraceuticals including: *Biological Modifying Polysaccharides, Direct-fed Microbials,* and *Phytonutrients.* Lastly, nutraceuticals are not regulated by the Food and Drug Administrations; therefore, statements regarding composition, dosage, effectiveness, and quality are not independently validated or standardized. This makes comparison and interpretation among extracts and/(or) commercials products difficult.

Biological Modifying Polysaccharides

Indigestible carbohydrates, including oligosaccharides and fructans, can improve health through a variety of mechanisms of actions. One of the mechanisms of action is through a symbiotic or prebiotic effect, where these indigestible carbohydrates are an energy source for probiotic bacteria and the health benefits are through improving the microbial ecology of the host gastro-intestinal tract. However, the focus of these indigestible carbohydrates in the current presentation will be on their immunomodulatory effects and ability to adsorb gram-negative bacteria as well as certain bacterial and fungal toxins.

Biological modifying polysaccharides are found in a variety of plants, milk, or the cell wall of fungi. We will focus on the polysaccharides extracted from cell walls of fungi, including mannan-oligosaccharides (MOS) and β-glucans (BG). The composition, availability, and physical chemistry of the extracts and carbohydrates influences their ability to improve the health of livestock. For example, a fungal extract from one fungus may behave very differently from another fungal extract. Further, many extracts are

blended with other ingredients; therefore, 1 gram of Product A may have a very different function than 1 gram of Product B. Unfortunately, the analytical chemistry to determine these structures and concentrations are complicated and expensive.

Type 1 fimbriae are mannose-specific filaments that are expressed on pathogenic gram-negative bacteria. These fimbriae on gram-negative bacteria were adsorbed by yeast cell wall extracts, and the ability to adsorb both *Salmonella sp.* and *E. coli* were correlated with the concentration of MOS (Ganner et al., 2013). The type 1 fimbriae adsorb to MOS and prevent the pathogenic bacteria from binding to the epithelium and colonizing the gastro-intestinal tract. Therefore, supplementing yeast extracts with greater quantities of MOS can be a useful prevention strategy for those animals with a greater exposure to gram-negative pathogenic bacteria (Davis, 2018). The impacts of MOS on specific immune responses is not well understood. Further, most fungal extracts that contain MOS also contain BG, which are known to have immunomodulatory effects.

B-glucans from cell wall fractions from fungal sources have the potential for immunomodulatory effects. The β-1,3 glucans are able to ligate Dectin-1 receptors on monocytes, macrophages, neutrophils and to a lesser extent on dendritic cells and T cell surfaces (Taylor et al., 2002). Further, the size of the BG extract influences both the leukocyte type and the response that is impacted (Elder et al., 2017). Data indicate that smaller BG, which are more common on less virulent fungi, typically impact innate leukocytes and may limit inflammation, whereas large BG oligosaccharides may increase inflammation. Oral supplementation of BG can also impact systemic immune responses in addition to local gastro-intestinal immune responses. The systemic effects could be both direct and indirect. The direct systemic effect is thought to be mediated by M-cells that sample intestinal lumen contents, including BG. Preliminary data from our laboratory indicate that dairy calves supplemented with a BG extract had increased relative abundance of larger oligosaccharides, 7 and 8 oligosaccharides, in peripheral circulation when compared to calves not supplemented BG (Davis and Ballou, unpublished).

Direct Fed Microbials

Direct-fed microbials, also known as probiotics, are classified as a live microorganism that can improve the health and/(or) performance of livestock. Common commercially available microorganisms include: *Lactobacillus sp.* and other lactic acid producing bacteria*, Bifidobacterium sp.*, *Bacillus sp.*, and *Saccharomyces cerevisiae*. The dose is commonly reported as the colony forming units supplemented per day or per kg of DM. Other important considerations when supplementing direct-fed microbials include the age or physiological state of the animal, infectious pressure, and the duration of supplementation. Oral supplementation of direct-fed microbials to impact gastro-intestinal health makes the most teleological sense because the supplemented microorganisms are targeting the microbial communities and cellular function within the gastro-intestinal tract.

The gastro-intestinal tract is a dynamic tissue that varies between animals, diets, age, environment, and management factors. To be considered a microorganism with probiotic effects it should have at least one of the following desirable outcomes:

- Regulate gastro-intestinal tract microbial communities
- Prevent adherence of potential pathogens in the gastro-intestinal tract
- Product anti-microbial or bactericidal molecules
- Improve gastro-intestinal tract integrity
- Improve mucosal adaptive immune responses
- Balance gastro-intestinal inflammation
- Improve fermentation and nutrient utilization

The application of direct-fed microbials in neonates is common. The gastro-intestinal tract of these animals is rapidly developing, and these animals are more susceptible to gastro-intestinal disease. Early in life the gastro-intestinal tract is colonized with facultative anaerobes, which includes many bacteria from the environment, and then shifts more toward strict anaerobes (Meale et al., 2017). Therefore, supplementing anaerobic lactic acid producing bacteria may speed up the microbial progression and reduce the risk for infection from environmental *Enterobacteriaceae* (Liang et al., 2020). The model of competitive exclusions has been around for a long time, where these more beneficial microorganisms are taking up space and utilizing nutrients that are then less available for disease-causing microorganisms. Further, lactic acid producing bacteria can help lower pH in the lumen of the gastro-intestinal tract, which can help limit the establishment of pathogenic *Enterobacteriaceae*.

Another mechanism through which direct-fed microbials can improve gastro-intestinal health is by modulating the gut-associated mucosal tissue immune system. Many immune factors concentrate themselves locally in the gastro-intestinal mucosa, including: secretory IgA, antimicrobial peptides, and other regulatory leukocyte responses. The immune factors are important to maintain gastro-intestinal integrity and function, as well as balance the local inflammatory response. Liang et al. (2020) supplemented Jersey bull calves with a blend of 2 strains of lactic acid producing bacteria and then challenged them with a moderate dose of *Salmonella enterica* serotype Typhimurium. The calves that were supplemented with the direct-fed microbials had reduced systemic and local inflammation after they were infected with the *Salmonella typhimurium.* Localized inflammatory responses are often considered beneficial in most tissues; however, in the gastro-intestinal tract an excessive or prolonged inflammatory response can further exacerbate the pathogenesis of the disease because of impaired gastro-intestinal integrity.

The use of direct-fed microbials in adult livestock are also used to support gastro-intestinal health. However, in adult ruminants many of the direct-fed microbials are fed to target the rumen and improve nutrient digestibility and utilization. Although the main target is the rumen, some of the direct-fed microbials can make their way through the rumen and have similar impacts on intestinal health as noted above for young calves. In fact, supplementing direct-fed microbials to feedlot cattle is a common industry practice for

preharvest food safety. Cattle supplemented with direct-fed microbials had decreased fecal shedding of pathogenic bacteria and decreased carcass contamination from the same bacreria (Brashears et al., 2003; Younts-Dahl et al., 2005; Peterson et al., 2007)In lactating dairy cows, most of the data focuses on production performance and milk quality. Fecal pathogen shedding or manure consistency are not often reported, but conceivably some of the performance benefits may be partially attributable to improvements in gastro-intestinal health. Further, the greatest health and economic benefits of supplementing direct-fed microbials are during stressful events, such as the transition period.

Phytonutrients

Phytonutrients are a broad group of compounds with potential therapeutic applications because they have antioxidative and anti-inflammatory properties. Plants synthesize polyphenols as a defense mechanism against both potential pathogens and ultraviolet irradiation. Various fruit and vegetable byproducts that are rich in phenolic compounds are available as feedstuffs for ruminants and may include citrus, grape, pomegranate, and green vegetable processing residues. Polyphenolic compounds can be absorbed in the small intestines and enter peripheral circulation where they can exert their bioactive effects on various tissue. The ruminal environment, like most of the dietary nutraceuticals discussed can modulate the activity of the dietary polyphenols. The rumen microbial communities can degrade polyphenols and decrease host availability. Therefore, the biological activity will depend on the structure in the diet as well as the concentration of the bioactive ingredients that bypass through the rumen.

Published reports on the immune effects of feeding flavonoid-rich products to ruminants are limited to predominately grape, pomegranate, and green tea derivatives. Supplementing grape polyphenols lowered leukocyte mRNA expression of superoxide dismutase in postpartum dairy cows (Colitti et al., 2006). Dairy calves supplemented with a pomegranate extract had increased *in vitro* secretion of interferon-γ and interleukin-4 as well as greater ovalbumin specific immunoglobulin G responses (Oliveira et al., 2010) Lastly, polyphenols from green tea extracts reduced the acute phase response of small ruminants following a parasitic challenge (Zhong et al., 2014). These data suggest that the antioxidative and anti-inflammatory properties of these polyphenolic-rich compounds can play a role in improving the health of livestock.

Essential oils are another class of phytonutrients. In addition to the immunomodulatory effects, some essential oils were reported to have an antimicrobial activity against food borne pathogens and rumen microorganisms. The majority of research on the immunomodulatory effects of essential oils was conducted in monogastrics. Some of the mechanisms of action are through a direct receptor-mediated improvement in mucosal blood flow, altered cytokine and neuropeptide release, or modified leukocyte function. In ruminants the main essential oils investigated were carvacrol and thymol from oregano oil, garlic, and capsaicinoids, but due to ruminal degradation the impacts of some of these supplements in mature ruminants are not well understood. Supplementing milk-fed dairy calves with oregano oil reduced the incidence of scours, improved hematology, and increased immunoglobulin concentrations of the

calves (Katsoulos et al., 2017; Seirafy and Sobhanirad, 2017; Ozkava et al., 2018). However, in lactating cows topical or intramammary administration of oregano failed to cure an experimentally-induced *Streptococcus uberis* infection. However, intra-abomasal infusion of garlic oil increased the neutrophil to lymphocyte ratio as well as increased the CD4 positive T cell population, and similarly, intra-abomasal infusion of capsaicinoids increased CD4 positive T cell proliferation (Oh et al., 2013). (Oh et al., 2013). Lastly, capsaicinoids were able to reduce the acute phase response to an intravenous lipopolysaccharide challenge in mature dairy cows (Oh et al., 2015).

Summary

Nutraceuticals are a diverse group of compounds. In order to be considered a nutraceutical the oral supplementation must improve some aspect of animal health and/(or) production efficiency. There remains a lot of ambiguity regarding nutraceuticals because this is a rapidly evolving field without a lot of regulatory oversight. The concentration of bioactive ingredients or compounds are often not known or reported. Further, a lot of commercial products are extracts which can contain many different bioactive compounds that may work in a symbiotic or opposing manner. The presentation discussed nutraceuticals as biological modifying polysaccharides, direct-fed microbials, and phytonutrients. These compounds work through a variety of mechanisms including stabilizing microbial communities, improving mucosal responses and barrier function, adsorbing potential pathogens or toxins, improving antioxidant status, direct antimicrobial activity, and either increasing or decreasing systemic leukocyte responses.

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Bacterial Inoculants for Optimizing Silage Quality and Preservation and Enhancing Animal Performance

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Introduction

Silage accounts for up to 60% of dairy cow diets in the US and approximately 133 million tons of corn silage alone were produced in 2019 (Adesogan et al., 2020). However, significant wastage of silage worth over $2 billion occurs annually in the US due to spoilage and other losses that range from 14 to 24% on average on farms (Rotz and Muck, 1994). One of the effective ways of curtailing such losses is applying bacterial inoculants to forages at the point of ensiling. Several excellent reviews have been published on silage additives including inoculants. These include those of McDonald et al. (1991), Muck and Kung (1997), Kung et al (2003) and Muck et al. (2018). Addah et al. (2014) also discussed the cost effectiveness of silage inoculants. Rather than another review, the intention in this paper is to briefly describe the types and modes of actions of various bacterial inoculants, to summarize the findings of different meta analytical studies (Table 1) on their use, and to describe critical factors for ensuring inoculant effectiveness.

Inoculant Effects on Silage Preservation and Quality

Homofermentative LAB

The use of bacterial inoculants for silage preservation has increased substantially over the last few decades with increasing reliance of silage in diets of dairy and beef cattle. Traditional bacterial inoculants were selected to improve silage preservation by fermenting plant water-soluble carbohydrates (WSC) into organic acids to inhibit the growth of deleterious bacteria and fungi, minimize dry matter (DM) losses and preserve important nutrients. Since these effects are predicated on rapidly acidifying the silage, the focus has been to select bacteria that efficiently dominate the epiphytic bacteria and ferment sugars into organic acids that rapidly decrease the pH. Homolactic fermentation involves conversion of one molecule of hexose into a single acid, lactic acid. In the silage context, obligate and certain facultative heterofermentative lactic acid bacteria (HoLAB) ferment one molecule of glucose into two molecules of lactate via pyruvate. This is the most efficient fermentation pathway as it results in minimal energy losses and no losses of CO_2, and hence no dry matter losses. Facultative heterofermentative bacteria can also ferment pentoses into lactic and acetic acid via the phosphoketolase pathway. Most bacterial inoculants used for silage making are HoLAB including those belonging to the *Lactobacillus, Enterococcus*, and *Pediococcus* groups such as *Lactobacillus acidophilus, L. casei, L. curvatus, L. paracasei, L. plantarum, L. salivarius, Lactococcus acidilactici, Enterococcus faecium, Pediococcus acidilactici and P. pentosaceus.*

Table 1. Meta analyses on effects of bacterial inoculation on silage quality parameters and animal performance

Studies	**Years of data collected**	**# of articles**	**Forage**	**LAB species**	**Application rate (cfu/g)**
Kleinschmit and Kung, 2006	1996 - 2005	23	corn, grass and small grain (barley, sorghum, wheat, ryegrass, and pea:wheat mixture)	*L. buchneri*	$\leq 10^5$ $> 10^5$
Oliveira et al., 2017	1997 - 2016	130	corn, sorghum, temperate grass, tropical grass, sugarcane, alfalfa, other legume; and other forages	*L. plantarum, P. pentosaceus*, *E. faecium*, *L. rhamnosus*, or mixed LAB species	$\leq 10^4$, 10^5, 10^6, $\geq 10^7$
Blajman et al., 2018	1980 - 2017	104	corn	*L. buchneri*, *L. plantarum, P. acidilactici*	10^4 - 10^7
Rabelo et al., 2018	2006 - 2016	42	sugarcane	*L. plantarum L. buchneri*	0 - 1.8 x10^6 for Lp 0 – 2.5 10^{10} for Lb
Zhang et al., 2018	2003 - 2013	24	corn	*Lactobacillus plantarum, L. buchneri, L. brevis, P. pentosaceous, E. Faecium, L. rhamnosus, L. lactis, L. acidilactici, L. acidophilus*	$< 10^5$; $\geq 10^5$
Bernardi et al., 2019	1980 - 2017	140	corn	*L. brevis, L. buchneri, L. acidophilus, L. curvatus, L. paracasei, L. plantarum, L. salivarius, E. faecium, P. acidilactici, P. pentosaceus*	4.3x10^2 - 6.7x10^{10} 1x10^3 - 7x10^8 3.4x10^4 - 1x10^8
Blajman et al., 2020	1980-April 2018	48	alfalfa	*L. buchneri, L. plantarum, P. acidilactici, E. faecium*	10^4 - 10^7
Arriola et al., 2020	1997-2018	120	whole-plant corn, whole-plant sorghum, temperate grass, tropical grass, sugarcane, alfalfa, other legumes, grain, high moisture corn, and other forages	*L. buchneri*, *L. plantarum, P. pentosaceus*, *E. faecium*, *L.hilgardii, L. casei, P. acidilactici, Lactococcus acidilactici*	$\leq 10^4$, 10^5, 10^6, $\geq 10^7$

In addition to using HoLAB to improve the fermentation, reduce DM losses, and preserve nutrients. increasing aerobic stability and digestibility are other desirable outcomes. To our knowledge, only one meta analytical study examined the efficacy of HoLAB at achieving these goals over a wide range of forage types, application rates and bacterial species. Based on 130 studies, Oliveira et al. (2017) showed that inoculation with HoLAB reduced silage pH and proteolysis and increased DM recovery of legume, tropical and temperate grass silages (Figure 1). This was achieved by increasing lactate production and reducing ammonia-N, acetate, and butyrate production. However, DM digestibility was not affected. No other studies have examined HoLAB effects across different forages, but some have examined specific forages. For instance, the meta-analysis of Bernardi et al., (2019) revealed that inoculating corn silage with HoLAB increased lactic acid concentration but still increased DM losses. Similarly, Rabelo et al. (2018) also showed that inoculating sugarcane silage with HoLAB (*L. plantarum* alone) increased DM losses. Collectively these studies suggest that applying HoLAB alone to corn, sorghum and sugarcane silages may not reduce DM recovery. This is probably because the latter forages have low buffering capacities and sufficient nonstructural carbohydrates to allow a natural homofermentative pathway to prevail even without inoculation. However, application of HoLAB to forages like alfalfa or grasses, that have lower nonstructural carbohydrate concentrations or high buffering capacities, improves fermentation, and reduces DM losses.

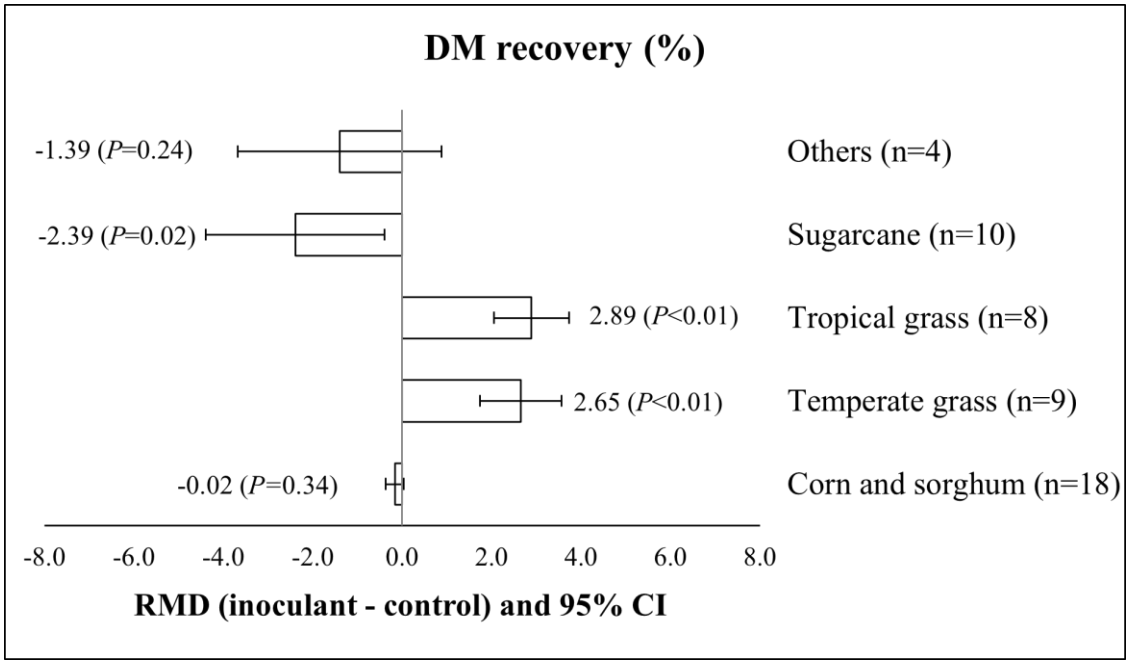

Figure 1. Effects of silage inoculation with homofermentative and facultative heterofermentative lactic acid bacteria (LAB) on silage DM recovery as affected by forage type. RMD = raw mean differences between inoculated and uninoculated treatments. Adapted from Oliveira et al. (2017).

The Oliveira et al. (2017) meta-analysis reported that HoLAB inoculants also reduced clostridia and mold growth but had no effect on aerobic stability. These results support the literature review of Muck and Kung (1997) and meta analyses on sugarcane (Rabelo et al., 2018) and corn (Bernardi et al., 2019) silages that had similar findings. The failure of HoLAB to reliably improve aerobic stability has led to development of combination inoculants that include heterofermentative bacteria (HeLAB), which increase aerobic stability, as well as HoLAB in inoculants.

Single Obligate Heterofermentative LAB

Obligate heterolactic bacteria ferment one molecule of hexose into lactic acid, carbon dioxide and ethanol. Following conversion of hexose into pyruvate, acetate and CO_2 via the pentose phosphate pathway, pyruvate and acetate are further reduced to lactate as well as ethanol and CO_2, respectively. The CO_2 and ethanol produced during heterofermentation contribute to DM losses, hence this pathway is less efficient than homofermentation. The main group of obligate heterolactic bacteria used in silage fermentation belong to the *Lactobacillus buchneri group,* among which *L. buchneri, L. brevis, L. diolivorans, L. hilgardii, L. kefiri, L. parafarraginis* have been tested on silage (Muck et al., 2018).

The most widely used obligate heterolactic bacterium is *Lactobacillus buchneri*, which is added to silage to reduce aerobic spoilage. This bacterium ferments lactic acid to acetic acid and 1, 2 propanediol (Oude Elferink et al., 2001). The 1,2 propanediol can be further converted to propionic acid by HeLab like *L. diolivorans* (Krooneman et al., 2002) or directly by a novel strain of *L buchneri* when glucose and cobalamine are present (*Lb. buchneri* A KKP 2047p; Zielinska et al., 2017; Muck et al., 2018). The acetic acid and propionic acid produced inhibit the growth of lactate-utilizing yeasts and molds that cause spoilage, thereby increasing silage aerobic stability. *Lactobacillus hilgardii*, has a similar mode of action *to L. buchneri* (Heinl et al., 2012) and some early studies suggest that it prevented DM losses (Avila et al., 2012) and increased aerobic stability relative to *L. buchneri* (Polukis et al., 2016). However, other studies have not shown clear and consistent differences in the aerobic stability response of both bacteria (Ferrero et al., 2018; Arriola et al., 2020a). Muck et al., 2018 cited several studies showing that antifungal compounds produced by *L. buchneri and* other HeLab may also contribute to improved aerobic stability.

The first meta-analysis on effects of *L. buchneri* alone on silage showed that the inoculant increased aerobic stability of silages in a dose-dependent manner (Kleinschmit and Kung, 2006). Applying *L. buchneri* at $\leq 10^5$ was less effective than $>10^5$ cfu/g at increasing the aerobic stability of corn, grass, and small grain silages. These authors confirmed that applying *L. buchneri* alone resulted in a small (1-1.8%) increase in DM loss. A recent meta-analysis by our group (Arriola et al., 2020b) confirmed that applying *L. buchneri* to various forages markedly increased aerobic stability (by 79%) except in tropical grasses and whole plant sorghum silages (Figure 2). This was confirmed in the meta-analysis of Blajman et al. (2018) on corn silage when *L. buchneri* or other unspecified individual obligate heterofermenters were examined. However, Rabelo et al.

(2018) showed that *L. buchneri* did not affect aerobic stability of sugarcane silage in their meta-analysis. The failure to increase aerobic stability of sorghum and sugarcane silages may reflect high residual WSC concentrations which led to ethanolic fermentations and the growth of lactate-assimilating yeasts that cause spoilage.

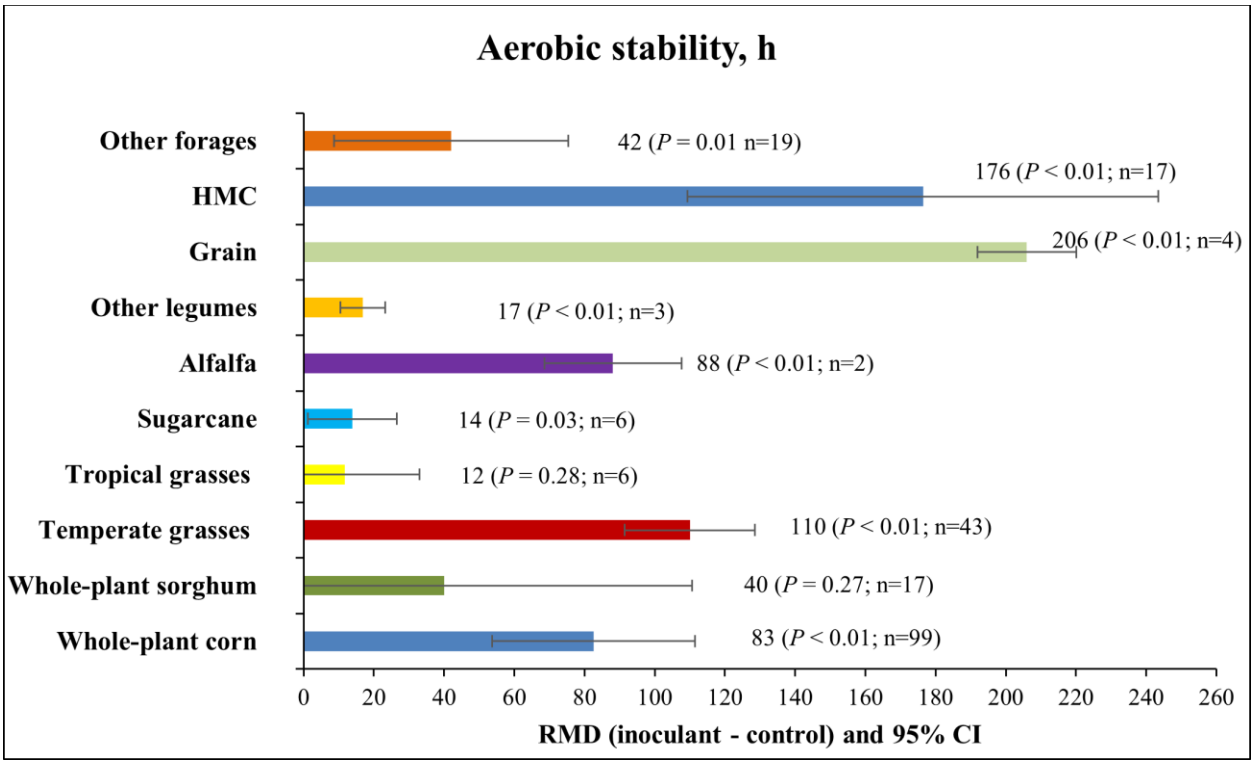

Figure 2. Aerobic stability responses to inoculation with *Lactobacillus buchneri* (LB)-based inoculants (LBB) with or without homofermentative or obligate heterofermentative bacteria as affected by forage type. RMD = raw mean differences between LB inoculated and uninoculated silage. HMC= high moisture corn; other forages= pea-wheat, rice, triticale, clover-ryegrass, alfalfa-ryegrass, oat, potato-wheat, sweet potato, potato hash. Adapted from Arriola et al. (2020b).

Lactobacillus buchneri increased DM losses by small amounts in meta analyses on corn and small grain silages (Kleinschmit and Kung, 2006; 1- 1.8%) and various forages (Arriola et al., 2020b; 0.3%; Figure 3). However, Rabelo et al. (2018) reported that *L. buchneri* application to sugarcane decreased ethanol production thereby reducing DM losses. Therefore, applying *L. buchneri* alone causes only small DM losses in most forages, and reduces them in sugarcane. There have been insufficient studies on obligate heterofermentative alternatives to *L. buchneri* like *L. hilgardii, L. brevis* (Danner et al., 2003), *L. kefiri* (Daniel et al., 2015) *or L. parafarraginis* (Liu et al., 2014) to determine their individual effects on aerobic stability with a meta-analysis.

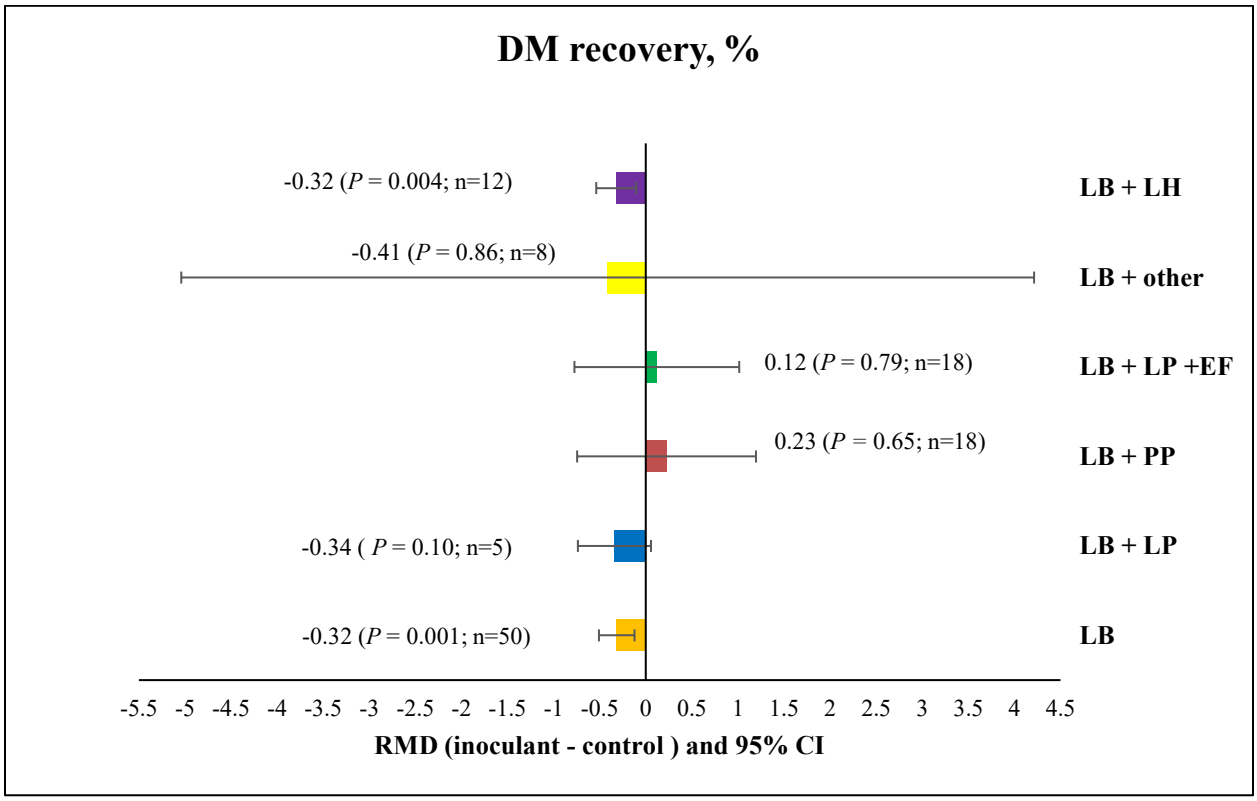

Figure 3. Lactic acid bacteria type effects on silage DM recovery responses to inoculation with *Lactobacillus buchneri* (LB)-based inoculants (LBB) with or without homofermentative or obligate heterofermentative bacteria. LB = *Lactobacillus buchneri* alone; LB+LP = *L. buchneri* with *Lactobacillus plantarum,* LB+PP = *L. buchneri* with *Pediococcous pentosaceus;* LB+LP+EF = *L. buchneri* with *L. plantarum and Enterococcus faecium;* LB+LP+PP = *L. buchneri with L. plantarum and P. pentosaceous;* LB+LH = *L. buchneri* with *Lactobacillus hilgardii*; LB+other = *L. buchneri* with other species like *Lactococcus lactis, Lactobacillus casei, Pediococcus acidilactici*. Adapted from Arriola et al. (2020b).

Obligate Heterofermentative LAB Mixtures

In the last decade, a few studies have examined combinations of *L. buchneri* with either *L. brevis*, *L. parafarraginis* or *L. hilgardii* (Avila et al., 2012, Liu et al., 2014; Ferrero et al., 2018, Arriola et al., 2020a). The purposes were to prevent DM losses and to achieve a greater aerobic stability than *L. buchneri.* A third purpose is to achieve aerobic stability earlier than *L. buchneri*, which requires 30 to 60 d to convert lactate to 1, 2 propanediol, and subsequently to increase aerobic stability (Muck et al., 2018). However, the results of applying mixtures of HeLab have been inconsistent. Our recent meta-analysis (Arriola et al., 2020b) showed that when a mixture of *L. buchneri and L. hilgardii* were applied to various forages, like *L. buchneri alone,* it markedly increased aerobic stability (79%) and slightly increased pH (0.7%) and DM losses (0.34%). These effects were achieved by

increases in acetate (47%), 1, 2 propanediol (269%) and propionate concentrations (35%), which decreased yeast counts as well as mold counts in most cases. Similar, reductions in yeast and mold counts and increases in acetate and aerobic stability were evident in the meta-analysis of HeLAB (*L. buchneri and L. brevis*) effects on corn silage but DM losses were surprisingly increased by 50% (Bernardi et al., 2019) partly due to greater losses in farm vs. laboratory silos.

Heterofermentative with Homofermentative LAB Mixtures

Most of the inoculant studies in the last decade have examined if applying HoLAB and HeLAB together (MixLAB) would capture the benefits of both types of bacteria while overcoming their drawbacks (Filya, 2003; Arriola et al., 2011). Specifically, the aim is to exploit the improvement in fermentation and reduction in DM losses by HoLAB, while overcoming their failure to improve aerobic stability by adding HeLAB. While these combination inoculants have included various HoLAB like *L. plantarum, E. Faecium, P. acidilactici, L. lactis*, almost all have included *L. buchneri* as the HeLAB, though *L. hilgardii or L. brevis* have been used in some instances.

Various meta analyses have examined effects of MixLAB on preservation of specific forages. Our recent meta-analysis (Arriola et al. 2020b) confirmed that across several forage types, they improved aerobic stability except in whole-plant sorghum and tropical grass silages, by reducing yeast and mold counts. In addition, they improved fermentation and prevented slight increases in DM losses and pH caused by applying HeLAB alone. Therefore, our study confirmed the multiple benefits of applying MixLAB cocktails.

Other meta analyses have also shown that aerobic stability was improved by applying MixLAB to corn (Blajman et al., 2018; Zhang et al., 2018; Bernardi et al., 2019) and alfalfa (Blajman et al., 2020) silage. This was attributed to acetate-mediated reductions in yeast and mold counts. Among the latter studies, MixLAB effects on DM losses were only reported by Bernardi et al. (2019), who noted that they were increased by 50% and by 23% by HeLAB and MixLAB, respectively. These increases are much greater than the slight increases reported by Kleinschmit and Kung (2006; 1-1.8%) and Arriola et al. (2020b; 0.3%) who only analyzed studies published in English. In contrast, the study of Bernardi et al. (2019) included older studies (1980 to 2017) published in English as well as Portuguese, and Spanish. Therefore, the higher DM losses in the Bernardi et al. (2019) study may partly reflect older responses as well as different management practices and forage species used in South America versus those in North America and Europe.

Inoculants Containing other Microbes

Studies have shown some promise in using alternatives to LAB for improving silage attributes such as *Propionibacteria* for improving aerobic bacteria (Filya et al., 2004), *Streptococcus bovis* for improving the fermentation (Ferreira et al. (2013) and *Bacillus* spp. for improving aerobic stability (Lara et al. (2016), etc. Studies have also

successfully inoculated forages with yeasts to preserve, multiply and deliver them to ruminants (Savage et al., 2014; Duniere et al., 2015). However, the number of studies using these LAB alternatives have been insufficient to verify their effects through a meta-analysis.

Inoculants Containing Digestibility Enhancers

Various enzymes have been added to certain inoculants to increase digestibility measures. Most of such studies have investigated effects of LAB inoculants containing "cellulase" or "hemicellulase" enzymes. These generic names do not specify the precise activities added and many studies have neither independently verified the enzyme activities nor examined effects of the bacteria with and without the enzyme. Thus, it is often impossible to ascertain if the enzyme improved digestibility. In the meta-analyses of Oliveira et al. (2017) and Bernardi et al. (2019), only 2.4% and 13% of 130 and 140 studies involved combinations of inoculants with enzymes, and enzyme inclusion had no effect on silage digestibility.

A few studies have examined the potential to use *L. buchneri* or *L. brevis* strains that improve aerobic stability but also improve secrete digestibility-enhancing esterase enzymes (Nsereko et al., 2008). However, while some of such attempts have shown promise, the results have not been consistent in corn (Kang et al., 2009; Lynch et al., 2015) or alfalfa silage (Lynch et al., 2014).

Combining HoLAB with sodium dodecyl sulphate (SDS), a surfactant, improved NDF degradability of barley silage whereas the inoculant alone only improved the fermentation (Baah et a., 2011). However, only a few of such studies exist and therefore their complementary effects on LAB have not been summarized through a meta-analysis.

Inoculant Effects on Animal Performance

Only three studies seem to have used a meta analytical approach to examine effects of inoculants on animal performance. We (Oliveira et al. (2017) examined effects of HoLAB on the performance of dairy cows and reported that across 31 studies, when at least 10^5 cfu/g were applied, milk production by dairy cattle was increased by HoLAB, regardless of the type of ensiled forage (Figure 4), LAB species and diet type but DM intake and DM digestibility were not affected. The increase in milk production was not evident when lower doses of HoLAB. In a subsequent meta-analysis of 12 studies, Arriola et al., (2020b) examined effects of HeLAB (*L. buchneri alone*) or MixLAB on the performance of dairy cows. Milk production, DM intake, DM digestibility and feed efficiency were not affected. In contrast, a meta-analysis of 35 studies on effects of applying different types of LAB to corn silage on the performance of sheep (16 studies) and beef and dairy cattle (25 studies) (Bernardi et al., 2019) reported that HoLAB or HeLAB did not affect milk yield ($P > 0.05$) but MixLAB decreased milk yield. They also reported that HoLAB increased DM intake in sheep, decreased it in beef cattle and increased in vitro DM digestibility and in vivo DM and NDF digestibility in sheep.

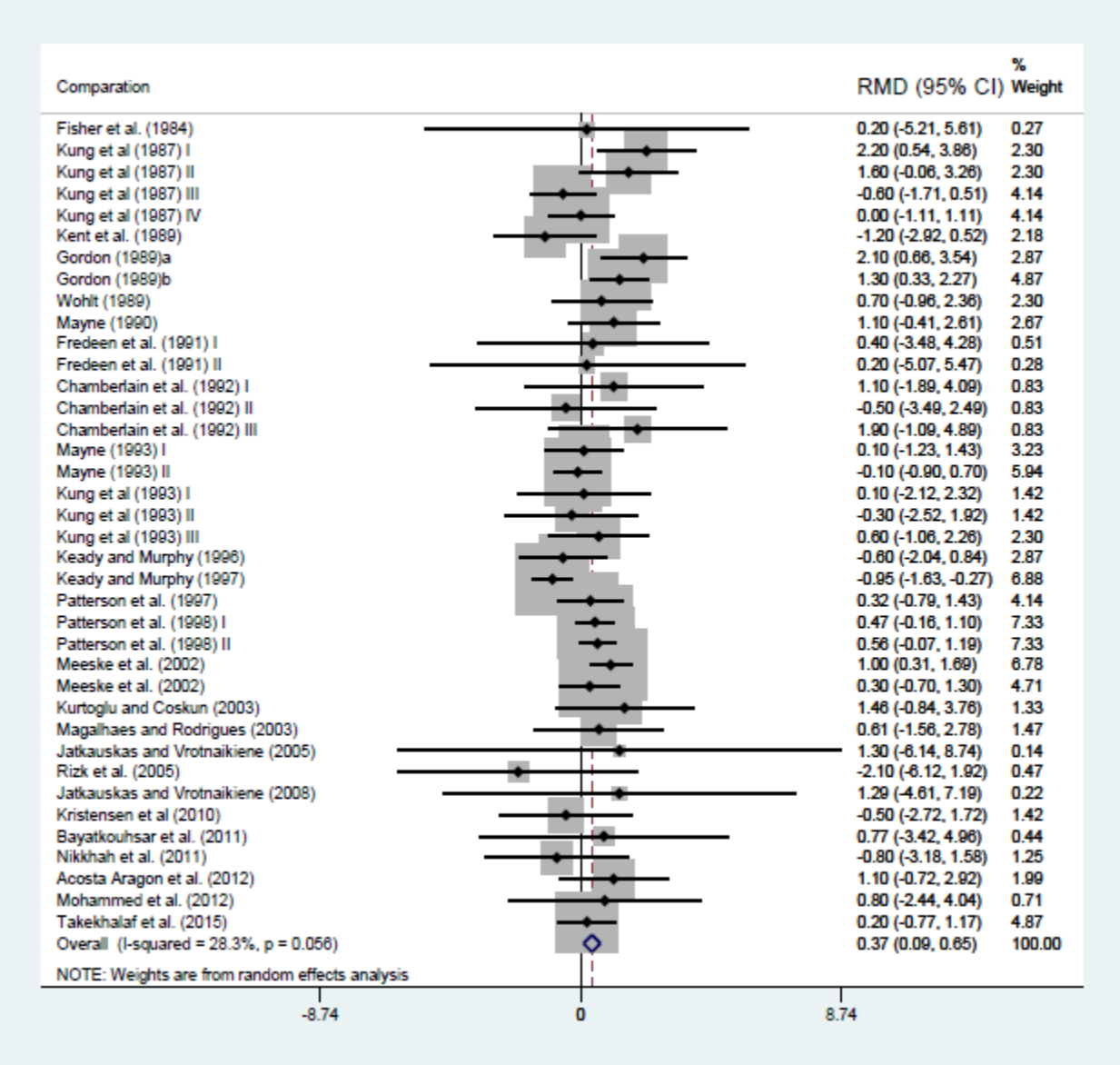

Figure 4. Forest plot showing the effect of silage inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on milk yield (kg/d) of dairy cows. The x-axis shows the raw mean difference (RMD); diamonds to the left of the solid line represent a reduction in the measure, whereas diamonds to the right of the line indicate an increase. Each diamond represents the mean size effect for that study, and the size of the diamond reflects the relative weighting of the study to the overall size effect estimate with larger diamonds representing greater weight. The lines connected to the diamond represents the upper and lower 95% confidence interval for the size effect. The dotted vertical line represents the overall size effect estimate. The diamond at the bottom represents the mean response across the studies, and the solid vertical line represents a mean difference of zero or no effect (Oliveira et al. (2017).

Differences between the dairy cow responses in our meta-analysis and that of Bernardi et al. (2019) may be due to the small number of studies involved as well as various factors pertaining to study inclusion and exclusion criteria. For instance, they included studies that were older (1983 – 2017), published in more languages (Portuguese, Spanish and English), and involved a wider range of HoLAB rates (10^4 to 10^{11} cfu/g) and more exotic cattle breeds. It can be surmised that based on the more recent studies (>1997) included in our meta-analysis, milk production is increased by HoLAB application but not by HeLAB or MixLAB. However, these trends may not be evident if older and non-English studies are included. Clearly more research is needed in this area to show if and how inoculation effects have change with time and geographical region.

Factors Affecting the Efficacy of Silage Inoculants

Several factors affect the efficacy of silage inoculants and thus influence the outcome of silage inoculant trials.

Inoculant Bacterial Composition

The bacterial composition is perhaps the most important factor influencing the efficacy of inoculants. The previous sections described the how silage fermentation pathways and products differ depending on the bacterial inoculant composition. Homofermentative bacteria should be added to improve the fermentation and DM recovery, not to improve aerobic stability. Heterofermentative bacteria be added to improve the aerobic stability not the fermentation. In addition, bacteria with the same fermentation pathway but with complementary pH niches may be combined in an inoculant. For instance, certain HoLAB combine *E. faecium or P. pentosaceus* as a starter culture with *L. plantarum* due to their ability to ferment hexoses at higher pH than *L. plantarum* (Kung, 2018). Bacteria strains also vary in efficacy; hence it is critical to select strains that have been proven in research studies.

Epiphytic Bacterial Population

Inoculants need to dominate the epiphytic bacterial population to shift the fermentation in the desired direction. McDonald et al. (1991) reported that a 10-fold domination of the epiphytic population by inoculant bacteria is required for their efficacy. Addah et al. (2014) noted that the population of inoculant LAB applied should be at least 10% greater than the natural bacteria that are on the forage. However, Lin et al. (1991) suggested that there was no relationship between epiphytic LAB numbers or species on adequacy of fermentation of alfalfa or corn silage. This disagreement may reflect the considerable variation in the types and numbers of epiphytic bacteria on different forages (100 to 1,000,000 cfu/g for alfalfa and barley and up to 1,000,000,000 cfu/g for corn; Bolsen et al., 1992; Merry and Davis, 1999; Addah et al., 2014). More research is needed to clarify the role of the species and population of epiphytic bacteria in the inoculant response.

Dose

Several authors have shown that inoculant effects on aerobic stability and DM losses, are dose dependent. Although doses examined in studies have ranged from < 10^4 to 10^{11} cfu/g, in practice the 10^4 to 10^6 cfu/g doses are most common. Most studies have shown that a dose of at least 10^5 cfu/g is necessary to reliably dominate the epiphytic bacterial population and produce the desired improvements in fermentation or aerobic stability (Kleinschmit and Kung, 2006; Oliveira et al., 2017). Higher doses ($\geq 10^6$ cfu/g) are sometimes more effective but may be uneconomical and lower doses are often less effective.

Bacterial Viability

Bacterial viability varies with prevailing conditions such as the moisture, temperature, acidity, etc. of the environment. Consequently, some manufacturers sell desiccants and oxygen scavengers with inoculants and recommend storing them in refrigerators prior to use. When testing inoculants, it is critical to verify the LAB counts and if necessary, adjust the dose, before inoculation. This is because some manufacturers add more bacteria than the recommended dose per bag to compensate for loss of viability prior to inoculation, while others add fewer. Exposure for several hours to the heat of the sun or to heat from the chopper engine can reduce viability of or kill inoculant bacteria, particularly after the inoculant is dissolved in water (Windle and Kung, 2016). This problem is more likely common when inoculant applicator tanks are close to the exhaust or engine of a chopper. Furthermore, inoculant viability may be reduced after 24 h of dissolution in water or by contaminants like chlorine or hydrogen peroxide in the water.

Mode of Application

Improper methods of inoculant application include those that involve manual application, shower-based methods, or application. These are all unlikely to be effective. Rather inoculants should be applied in a fine spray during chopping in the field to ensure uniform distribution throughout the forage mass. Proper calibration of inoculant applicators several times on the day of application critical.

Forage Type and Characteristics

The meta-analyses of Kleinschmit and Kung (2006), Oliveira et al. (2017) and Arriola et al. (2020b) revealed forage-specific effects of HoLAB and HeLAB on silage DM losses and or aerobic stability. HoLAB are more likely to be effective in forages that have high buffering capacities, high DM concentrations and low WSC concentrations and less effective in those that are too wet or immature at harvest. Fermentation is less likely to be improved by HoLAB application to well managed whole plant corn, sugarcane, or sorghum silage.

Silage Management

Delayed sealing may also reduce the rate of acidification of silage and it increases DM and energy losses. In addition, other poor silage management practices like poor sealing, low packing density, and low feedout rate can allow proliferation of spoilage yeasts and molds that increase aerobic stability (Borreani et al., 2018). Inoculant application may be more effective with these scenarios, but this should not be considered as an excuse for bad management.

Take Home Messages

1. Bacterial inoculants can be used to improve the fermentation, DM recovery, and aerobic stability of silage and the performance of dairy cows.

2. Inoculant effects vary with the species, strain and inoculation rate of the bacteria, the forage type and attributes and the storage and application method.

3. Homofermentative inoculants should be selected to improve the fermentation and reduce losses of DM, energy, and nutrients. They are particularly effective in forages with high buffering capacity or low WSC concentration and have improved milk production by dairy cows when applied at 10^5 cfu/g or greater.

4. Heterofermentative inoculants inhibit yeast and mold growth thereby increasing aerobic stability. They may cause a small increase in DM losses that is often offset by the improved aerobic stability, but they do not typically affect animal performance.

5. Combinations of homofermentative and heterofermentative bacteria may improve the fermentation, avoid, or minimize DM losses and improve aerobic stability.

6. To ensure efficacy, research-proven inoculants should be selected. They should be stored in a cool dry location and applied as recommended by the manufacturer at $\geq 10^5$ cfu/g.

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Impact of Daily and Seasonal Rhythms in Maximizing Milk Production

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Introduction

Biological rhythms are repeating patterns that are driven by time-keeping mechanisms within the animal and are adaptive as they coordinate physiology and metabolism with the external environment. The dairy cow has a well-recognized natural daily pattern of feed intake and milk synthesis and an annual rhythm of milk composition, but regulation of these rhythms has not been well described in the literature or well considered in current dairy management. We commonly assume that feeding a total mixed ration creates constant ruminal conditions, but the large variation in the rate of feed intake across the day causes large fluctuations in rumen fermentation and absorbed nutrients. Milk composition also differs across the day due to both dynamics in nutrient absorption and biological regulation attempting to match milk yield and composition with calf requirements across the day. Additionally, the consistent decline in milk yield and fat and protein concentration during the summer is often thought to be because of heat stress. While heat abatement strategies are very important for maintaining health and productivity of dairy cows in the summer months, evidence suggests that summer declines in likely due to cows' inherent annual rhythms. First, it is important to consider daily and seasonal rhythms while setting goals and evaluating herd production. Managing feeding times provides the opportunity to modify feed intake across the day, but behavior responses are complex. It is not entirely clear how to overcome seasonal rhythms, although appropriately managing photoperiod is recommended.

Background

Rather than simply responding to an environmental stimulus, endogenous timekeepers in the hypothalamus allow the animal to anticipate daily and yearly environmental changes before they occur. The timekeepers create rhythms that then drives adaptive changes in metabolism and physiology that increase survival. Two important aspects are the timing of the rhythms are set or "entrained" by environmental signals, such as light dark cycles, and the rhythms will persist if the animal is held under constant conditions because it is running within the body. Two major rhythms of importance to the dairy cow are circadian and annual rhythms.

Circadian Rhythms

Circadian rhythms refer to 24-hour repeating cycles followed by most physiological functions. Circadian rhythms are created by endogenous timekeeping mechanisms and are adaptive as they temporally coordinate behaviors and physiological processes with daily changes in the environment. Anyone who has flown across time zones or lost a night of sleep, or even just changed clocks to daylight savings time, appreciates the

physiological and psychological importance of circadian rhythms. Their importance is also strongly supported by scientific evidence. For example, epidemiological data in humans clearly shows that disruption of circadian rhythms by night-shift work increases mortality and morbidity and is especially associated with many conditions normally associated with stress.

The “biological clocks” that keep track of what time it is exist in most tissues in the body. The biological clocks in metabolically important tissues (e.g. adipose and liver) are responsive both to timing of light-dark cycles that controls the master clock in the brain, but also the timing of food availability. Interestingly, in experimental models the timing of food intake can alter the synchrony between the central master timekeeper and peripheral clocks, resulting in development of numerous disorders including obesity, insulin resistance, and metabolic diseases (Reviewed by Takahashi et al., 2008). We have demonstrated that there is a biological clock in the mammary gland that responsive to the timing of feed intake.

Daily pattern of feed intake

Feeding behavior is centrally regulated through integration of many factors including hunger, satiety, physiological state, environment, and endogenous circadian rhythms (Allen et al., 2005). Grazing cows have a well described “crepuscular” feeding pattern with a large proportion of intake consumed at dawn and dusk (Reviewed by Albright, 1993). It is important to remember ruminants are prey animals and daily feeding patterns are expected to have been impacted evolutionarily by changes in risk of predators and nutritional value of forages over the day. Importantly, pasture forages are highest in sugar and amino acids in the afternoon after photosynthesis has occurred. A circadian rhythm of intake with greater intake during the afternoon synchronizes hunger with maximal forage quality.

Using an automated observation system, we have observed the effect of feeding time and diet composition on the daily rhythm of intake. The daily pattern of intake in high producing cows and the effect of feeding time is well illustrated in an experiment where we fed cows 1x/d at 0830 h or 2030 h (Niu et al., 2014). Over 20 and 34% of daily intake was consumed in the 2 h after feeding in cows fed at 0830 and 2030 h, respectively. The intake rate at other times of day did not differ greatly, with both groups having lower intake overnight and higher intake in the afternoon. Before this work we commonly thought that cows consumed feed mostly during the day because that is when we delivered feed and it was the freshest. Delivery of fresh feed is a strong stimulus for feed intake. However, it is interesting to note that cows fed in the evening had low intake during the overnight (not different from morning fed cows) and waited till the following afternoon when feed was over 16 h old to increase intake to about twice that of the overnight period. This experiment highlights that cows have a strong natural drive to consume feed during the afternoon and early evening and timing of feed delivery is a strong stimulant to modify this pattern and has been replicated in other studies.

Physiological significance of the circadian pattern of intake

The ruminant has a rather consistent absorption of nutrients over the day because of more frequent meals, the size of the rumen, and the slow rate of ruminal digestion. However, highly fermentable diets are commonly fed to maximize energy intake and microbial protein production and result in a rapid production of volatile fatty acids (VFA) after consumption (Allen, 1997). Additionally, differences in the rate of feed intake over the day results in a large difference in the amount of fermentable substrate entering the rumen over the day.

The dynamic nature of rumen fermentation throughout the day is supported by high resolution observations of rumen pH by our lab and others (e.g. Yang and Beauchemin, 2006;DeVries et al., 2007;Harvatine, 2012), which clearly show a daily pattern of rumen pH with a nadir approximately 10 h after feeding. We also observed that ruminal digesta weight and starch concentration were 24% and 87% higher, respectively, 4 h after feeding compared to 1.5 before feeding. Additionally, we have observed that ruminal starch and NDF concentration over the day fit a cosine function with a 24 h period demonstrating a daily rhythm (Ying et al., 2015). We are not aware of a characterization of the rate or composition of duodenal flow throughout the day, but a daily rhythm has also been reported for fecal particle size, neutral detergent fiber (NDF), indigestible NDF, and starch concentration (Maulfair et al., 2011). We have also observed that the rhythm of fecal NDF was dependent on the time of feeding (Niu et al., 2014). Taken together, there is strong support for a circadian rhythm of nutrient absorption.

Evidence of circadian regulation of milk synthesis

Dairymen commonly recognize that morning and evening milking differ in milk yield and composition. Quist et al. (2008) conducted a survey of the milking-to-milking variation in milk yield and composition on 16 dairy farms. Milk yield and milk fat concentration showed a clear repeated daily pattern over the 5 days of observation in herds that milked 2 and 3 x/d. We have also observed milk yield and milk composition at each milking while milking every 6 h and feeding cows 1 x/d at 0800 h or in 4 equal feedings every 6 h (Rottman-Gredell et al., 2014). This demonstrated the daily pattern of milk synthesis in cows and identified an interaction with the timing of feed intake. We have further demonstrated shifts in the timing of milk synthesis through fasting cows for a short period during the day compared to the night (Salfer and Harvatine, 2020).

Recent work at Purdue tested the effect of light-dark phase shifting on metabolic health in transition dairy cows (Suarez-Trujillo et al., 2020). They observed that light phase shifting reduced the circadian rhythms of core body temperature and melatonin. Phase shifted cows also had increased total resting time, but decreased resting bout durations. Phase shifted cows did increase milk yield 2.8 kg/d over the first 60 d of lactation, although this may be due to a change in nutrient partitioning and the long-term effect was not investigated.

Lastly, automated milking systems (AMS) provide an opportunity to observe a natural preference for milking time. Care is needed in interpretation of cow behavior in AMS because of the confounding factors of demand for the robot and the entrainment by multiple factors. However, the frequency of cows entering the milking system appears to follow a circadian pattern (e.g. Hogeveen et al., 2001;Wagner-Storch and Palmer, 2003). For example, Wagner-Storch et al. (2003) reported 2% of cows in the holding area between 0000 and 0500 h compared to 8 to 12% of cow between 0800 and 1900 h. The preference for milking time may be due to a natural circadian synchronization with environmental factors or simply support a natural low activity period of the day.

Annual Rhythms in the Dairy Cow

Annual rhythms are present in nearly all studied organisms as a mechanism to perceive and adapt to seasonal environmental changes. For example, many mammals in northern climates hibernate over the winter and birds undergo major metabolic adaptations preparing for and during migration. Similar to circadian rhythms, these annual changes in physiological persist even after animals are placed in constant day length (photoperiod). This indicates that the rhythm is internally generated and not simply a direct response to environmental factors, although the timing of the internal rhythm is entrained or modified by changes in the environment across the year.

Annual Rhythms of Milk Yield and Composition

Yearly patterns of milk production have been recognized for over 40 years (Wood, 1970). Producers are familiar with summer declines in milk production, and recovery during the winter. When examining average monthly bulk tank records from U.S. Federal Milk Marketing Orders, the presence of an annual rhythm is apparent as we recently reported (Salfer et al., 2019). Fat and protein concentration display repeating 12-month cycles that are remarkably consistent between years. These yearly patterns fit a robust cosine function, suggesting that they represent a biological rhythm. The rhythms of fat concentration peak between December and January in most regions. Protein concentration is even more consistent between regions, with a maximum around the first of the year. The variation in milk fat concentration due to the annual rhythm is between 0.14 and 0.28 percentage units across the year, depending on the region. Notably, annual rhythms of fat concentration in the southern U.S. milk markets, mainly Florida and Arizona-Las Vegas, have lower amplitude rhythms than more north regions. The amplitudes of milk protein concentration were more consistent between regions, with peak to trough difference being 0.16 to 0.20 percentage units.

The presence of yearly rhythms in milk component yield was also characterized using ten years of DHIA data from individual herds in Minnesota, Pennsylvania, Texas and Florida (Salfer et al., 2020). Similar to the U.S. milk markets, milk fat and protein concentration peaked in December and January. Milk yield also followed an annual rhythm with peak milk yield occurring in April and lowest milk yield in September. There was a larger amplitude of the milk yield rhythm in FL and TX. Milk fat and protein yield are driven both by milk composition and yield and different timing and amplitudes of the

rhythms resulted in peak milk fat and protein yield occurring in late February and early March. Additionally, there was a larger change in milk fat and protein yield in FL and TX than MN and PA. Overall, the rhythm of milk yield aligned with the equinoxes while milk composition aligned with the solstices, indicating presence of two seasonal time keeping mechanisms.

We have also explored other cow factors that might impact the seasonal rhythms. The rhythm is similar between first lactation and multiparous cows (Salfer et al., 2019). The diacylglycerol o-acyltransferase 1 (DGAT1) gene, responsible for 40% of the genetic variation in fat percentage (Winter et al., 2002), also does not influence annual rhythms of fat concentration or fat yield (Salfer et al., 2019). There appears to be slight differences in the annual rhythms between breeds (Salfer et al., 2020). The timing of the rhythm was very similar, but the amplitude of fat and protein concentration was slight lower in Holsteins. While it is difficult to determine if this is due to genetic differences between breeds or herd management, the small differences should be accounted for when predicting production.

Naturally, environmental temperature is often blamed for causing the seasonal changes in milk production. While heat stress certainly impacts production, our results suggest that an annual rhythm exists independent of temperature (Salfer et al., 2020). Briefly, the model containing the seasonal rhythm fit better than the model testing the effect of daily maximum temperature. Furthermore, a decline in fat and protein concentration is observed below the fitted cosine function in July and August, especially in Pennsylvania and Minnesota. This phenomenon appears to suggest that heat stress is an additive effect, separate from the annual rhythm that causes additional production declines in the summer. It is also important to note that milk yield reaches a minimum in late September, instead of during the middle of the summer when temperatures are the highest. Lastly, experimental induction of heat stress results in decreased milk yield and increased milk fat concentration, which is opposite of that observed during the summer.

Potential Mechanisms of Seasonality

A primary role of annual rhythms is to coordinate reproduction with food availability to maximize the likelihood of survival of the offspring. As a component of reproduction, it is not unexpected that lactation would be controlled through similar mechanisms. Producing more energy-dense milk with greater concentrations of fat and protein in the winter when energetic demands are greater would increase the likelihood of calf survival. In all mammalian species characterized, annual rhythms are controlled by a photoperiodic timer based on the duration of melatonin release. The synthesis of prolactin is also under the control of the photoperiod-based mechanism. Prolactin is released from the pituitary and is involved in feed intake and initiation of lactation in many mammalian species (Bauman and Currie, 1980).

Effects of Photoperiod on Milk Production

Extensive research has examined the impact of altering photoperiod length on milk synthesis of the dairy cow. The first report of increased milk production after 16 h light: 8 h dark (16L:8D) photoperiod was make by Dr. Tucker's lab at Michigan State (Peters et al., 1978). Since this initial discovery, several subsequent experiments have confirmed these findings (Dahl et al., 2000;Dahl et al., 2012). The effect occurs after implementation of any photoperiod greater than 12L: 12D, however the response is greatest at 16L: 8D.

The results of photoperiod experiments and annual rhythms of production are seemingly at odds. While long-day lighting consistently increases milk synthesis, the cow's natural annual rhythm has decreasing milk yield throughout the summer and peak milk yield near the spring equinox. One potential explanation is that long-day lighting may induce photorefractoriness to the annual rhythm of milk synthesis. Photorefractoriness is a phenomenon observed in other species where long-term exposure to a constant photoperiod leads to spontaneous reversion of a seasonal physiological response to the state expected in the opposite photoperiod (Lincoln et al., 2005). In other species, a fixed photoperiod must be applied for a long period of time (4 to 12 weeks) before switching of the physiological response occurs. In cows, the increase in milk yield after long days typically does not manifest until after 4 weeks of administration (Dahl et al., 2000). While this mechanism seems promising as a possible explanation for the observed effects of long-day lighting, it has not yet been studied in cows and further research must be done to test if it is related to the milk yield response.

Take Home Messages

- "Biological clocks" within the cow are keeping track of what time of day and what day of the year it is and create daily and annual rhythms. This robust system coordinates physiology and metabolism with the external environment.
- The dairy cow has a clear daily pattern of feed intake and milk synthesis. The timing of feed delivery and feed management are our best opportunities to modify this daily pattern.
- There is a seasonal pattern of milk yield and composition that is independent of heat stress. Managing lighting is probably our best opportunity to modify the seasonal rhythm.
- The daily and annual rhythms of milk yield and composition should be considered when setting goals and evaluating herd performance.

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Relationships between Starch and Physically Effective and Undegraded Fiber in Lactating Dairy Cows

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Introduction

For several years we have been working at the Institute on how to integrate measures of fiber (un)degradability and particle size in an effort to better predict dry matter intake (DMI) and energy-corrected milk (ECM) production (Grant et al., 2018). To-date, we have focused mainly on physically effective neutral detergent fiber (peNDF) and undegradable NDF at 240 hours of in vitro fermentation (uNDF240). The resulting value – termed physically effective uNDF240 (peuNDF240) - can be calculated simply as the physical effectiveness factor (pef) multiplied by uNDF240, or perhaps more accurately over a wide range of diets, as a direct in vitro measure of uNDF240 on the pef fraction of particles (more about this topic later). The pef is measured by sieving the total mixed ration (TMR) sample: either using a 1.18-mm sieve when dry, vertical sieving (Mertens, 1997) or using a 4.0-mm sieve when horizontally sieving as-fed samples on the farm. At least for corn silage and haycrop silage-based TMR, using the Penn State Particle Separator with a 4.0-mm sieve yields similar pef values as the standard dry sieving method with a 1.18-mm sieve (Schuling et al., 2015).

The objectives of this paper are to briefly review the progress to-date on integrating pef and uNDF240 to better predict DMI and ECM, and to present the lactation results from a recently completed study that investigated the interaction between dietary peuNDF240 and rumen fermentable starch (RFS).

Physically Effective Undegradable NDF

Miller et al. (2020) assembled a 5-study database from experiments using high-producing Holstein dairy cows at Miner Institute conducted between 2014 and 2019 to assess the relationship between uNDF240 and peuNDF240 with DMI and ECM. Details are provided in the abstract and the accompanying presentation from the 2020 American Dairy Science Association (ADSA) virtual annual conference (https://virtual2020.adsa.org/). Within this database, the range in dietary uNDF240 was 5.5 to 11.5% of ration dry matter (DM) and the range in peuNDF240 was 4.0 to 7.3 % of DM. This range in NDF undegradability spans what is commonly fed in the US with values of 10.0 to 11.5% more likely to limit DMI and values closer to 5 to 6% increasing the risk for subacute ruminal acidosis.

The relationship between uNDF240 and DMI (lb/d) was moderate ($y = -0.84x + 68.18$, $R^2 = 0.32$), but the relationship between peuNDF240 and DMI was stronger ($y = -2.16x + 72.42$, $R^2 = 0.60$). In particular, combining pef and uNDF240 allowed a better

prediction of DMI when higher uNDF240 diets were more finely chopped. Our research to-date suggests that when forage fiber digestibility is lower than desired, a finer forage particle size will enhance DMI and ECM production. The improved lactational performance appears to be associated with less eating time and a more desirable rumen fermentation and fiber turnover for cows fed higher uNDF240 diet with finer chop length.

The relationship between uNDF240 and ECM (lb/d) was strong (y = -2.26x + 126.38, R^2 = 0.58), but similar to DMI, the relationship between peuNDF240 and ECM (lb/d) was even stronger than that observed for uNDF240 (y = -4.92x + 133.14, R^2 = 0.78). A field study reported by Geiser and Goeser (2019) using 55 commercial dairy farms where corn silage comprised 36.8 ± 7.9% of the ration DM found that a one-unit increase in uNDF240 of the corn silage was associated with a 0.59 lb/d decrease in DMI and a 1.30 lb/d reduction in ECM. In the Institute data base, we observed a reduction of 0.84 lb/d of DMI and 2.3 lb/d of ECM with each one-unit increase in ration uNDF240 with high-producing cows (Miller et al., 2020). So, there is general agreement between our Institute database and this field study which gives us confidence that these relationships are consistent and can be useful in the field.

We need to note that the diets in this database were primarily based on corn silage and haycrop silage with some chopped hay and straw. Importantly, there were no pure alfalfa diets, diets with larger amounts of non-forage fiber sources, or pasture. In the future, we intend to define the relationships between uNDF240, peuNDF240, and DMI and ECM for a wider range of diets and management scenarios. Nonetheless, there appears to be value in integrating two measures of fiber - uNDF240 and pef – when formulating rations.

Interactions between Physically Effective uNDF240 and Rumen Fermentable Starch

Our most recent work has evaluated the relationship between dietary peuNDF240 and RFS (Smith et al., 2020). Initial studies were focused mainly on the middle to upper range of dietary uNDF240 concentrations to determine at what point DMI was constrained and how manipulating particle size affected DMI at a given uNDF240 content (Grant et al., 2018). In contrast, the study by Smith et al. (2020) was designed to determine the interaction between dietary starch (specifically RFS) and uNDF240 for diets that were on the lower end of the uNDF240 range commonly observed in the field. Consequently, the research focus shifted from gut fill and DMI constraints to maintenance of adequate dietary fiber and minimizing the risk of subacute rumen acidosis.

The negative associative effect of starch on rumen fiber degradation and peNDF requirements is well known. Mertens and Loften (1980) were the first to observe that too much starch resulted in lengthened lag times prior to NDF degradation in vitro. Subsequent work showed that, as rumen starch fermentability increased, the negative effect on the lag and fractional rate of NDF degradation increased and lower rumen pH amplified this negative effect of starch (Grant and Mertens, 1992; Grant, 1994). However, we still need to understand how dietary starch content and RFS influence rumen NDF

turnover in diets that differ in their fiber characteristics such as uNDF240, peuNDF240, and fast- and slow-degrading NDF (measured using 30-, 120-, and 240-h in vitro fermentations).

Details of the study by Smith et al. (2020) are available in the abstract and at the ADSA annual conference web site. Briefly, 16 lactating Holstein cows (8 ruminally fistulated) that were approximately 85 ± 15 days in milk were enrolled, blocked by parity, days in milk, and milk production and were used in a replicated 4 x 4 Latin Square design. The study had 28-d periods (18 d of adaptation, 10 d of collection). A factorial arrangement of four diets was used to evaluate the effect of dietary peuNDF240 content, dietary RFS content, and their interaction. Table 1 lists the primary dietary ingredients that were used in the study. Differences in dietary uNDF240 or peuNDF240 content were obtained by using a brown midrib (lower peuNDF240 diets) versus a conventional corn silage hybrid (higher peuNDF240 diets). The two dietary RFS concentrations were obtained primarily by varying the content of finely ground corn meal together with the starch in the corn silages. The corn meal contained 62% of DM ≤ 0.60 mm when dry sieved with a pef = 0.10.

Table 1. Ingredient composition of diets with varying concentrations of physically effective 240-h undegraded neutral detergent fiber (peuNDF240) and ruminal fermentable starch (RFS).

	Diets			
	Low peuNDF240		High peuNDF240	
Ingredients, % of DM	Low RFS	High RFS	Low RFS	High RFS
Conventional corn silage	-	-	47.60	47.60
Brown midrib corn silage	47.60	47.60	-	-
Timothy hay, chopped	7.94	7.94	7.94	7.94
Wheat straw, chopped	1.59	1.59	1.59	1.59
Corn meal	2.78	7.94	3.57	8.73
Beet pulp pellets	7.14	5.16	6.35	4.37
Concentrate mix	32.95	29.77	32.95	29.77

Table 2 summarizes the chemical composition of the four treatment diets. Unexpectedly, the two corn silage hybrids did not differ as much as anticipated in their uNDF240 content as they were fed out during the trial: 8.6% of DM for conventional versus 6.7% of DM for the brown midrib corn silage (although initial samples used in ration formulation had indicated 11.8% and 5.6% of DM for conventional and brown midrib, respectively). Consequently, the dietary uNDF240 concentration averaged 6.85% of ration DM for the lower uNDF240 diets and 7.20% of DM for the higher uNDF240 diets; in other words, the uNDF240 content was quite similar across all diets. Similarly, the peuNDF240 values (pef x uNDF240) were similar and ranged from 3.88 to 4.16% of ration DM. For all diets, the uNDF240 and the peuNDF240 values were on the lower end of the range in our 5-study data base.

Because the cows responded to dietary fiber characteristics (see Tables 3 and 4), and yet the measured uNDF240 and calculated peuNDF240 (pef x uNDF240) values did not differ markedly, we decided to directly measure the uNDF240 concentration (using an in vitro fermentation) in the fraction of each diet that was retained on the ≥1.18-mm sieve and the fraction that passed through this sieve. Interestingly, the uNDF240 was not uniformly distributed across the two size fractions as had been the case in some previous research (Grant et al., 2018). The directly assayed peuNDF240 averaged 6.2 and 8.3% of ration DM for the lower peuNDF240 and higher peuNDF240 diets, respectively. This range in directly measured peuNDF240 helps to explain the animal responses in Table 3 and 4. However, it does call into question the validity of simply calculating peuNDF240 as pef x uNDF240 in all dietary scenarios. In many instances, this simple approach appears to work well, but we need to be aware that, if the uNDF240 is not uniformly distributed across the particle size fractions, then the calculated number may not be appropriate. In addition, we need to be specific about how the peuNDF240 is measured. In this article, we will use the terms *calculated* or *assayed* peuNDF240.

The dietary starch content averaged 20.7 and 24.7% of DM for the high and low RFS diets, respectively. Starch degradability did not differ much across diets, but the RFS content averaged 16.8 and 19.1% of ration DM for the lower and higher RFS diets, respectively. It is important to put these starch measures into context. Although the diets differed by 4 units in starch percentage, the starch and RFS contents were moderate to low compared with many commonly fed diets in much of the US. The fact that the higher RFS diets were only moderately high is important to consider when interpreting the animal responses where negative effects on milk fat percentage were observed with relatively low RFS concentrations (see Table 4). Assessment of the interaction between RFS and fiber may be especially important with lower fiber diets with increased risk of subacute rumen acidosis (pH < 5.8; Stone, 2004).

Finally, a post-hoc analysis of the intake of dietary carbohydrate fractions was performed using Cornell Net Carbohydrate Protein System (CNCPS) biology (NDS Professional, CNCPS biology v. 6.5, Reggio Emilia, IT) with Kurt Cotanch (Barn Swallow Consulting, LLC, Underhill, VT). This analysis used the ingredient compositional measures and animal measures from the study. Intake of uNDF240 was 2.2, 2.2, 2.5, and 2.4 kg/d for the lower peuNDF240/lower RFS, lower peuNDF240/higher RFS, higher peuNDF240/lower RFS, and higher peuNDF240/higher RFS diets, respectively. In the same dietary order, the intake of RFS was 5.0, 5.6, 5.0, and 5.5 kg/d. The ratio of dietary RFS:uNDF240 was 2.42, 2.82, 2.32, and 2.68 which may potentially have usefulness as a benchmark for milk fat depression (see discussion for Table 4).

Table 2. Composition of diets with varying concentrations of physically effective undegraded neutral detergent fiber after 240-h fermentation (peuNDF240) and rumen fermentable starch (RFS).

Item	Diets			
	Low peuNDF240		High peuNDF240	
	Low RFS	High RFS	Low RFS	High RFS
Dry matter (DM), %	55.3	55.3	54.4	54.2
Crude protein (CP), % of DM	16.1	15.3	16.0	15.2
Soluble protein, % of CP	40.6	39.8	43.4	42.5
aNDFom[1], % of DM	33.1	32.4	33.3	32.6
Lignin, % of DM	3.21	3.1	3.5	3.42
Starch, % of DM	20.7	24.6	20.8	24.7
Starch degradability[2], % of starch	80.5	78.1	81.4	77.0
Rumen fermentable starch, % of DM[3]	16.7	19.2	16.9	19.0
Sugar, % of DM	3.9	4.5	4.7	4.5
Ether extract, % of DM	3.83	3.76	3.81	3.75
uNDF30om, % of DM	13.5	15.2	15.1	15.5
uNDF120om, % of DM	7.5	7.6	8.5	8.5
uNDF240om, % of DM	6.9	6.8	7.3	7.1
pef[4]	0.60	0.57	0.57	0.57
Calculated peuNDF240 (pef x uNDF240), % of DM	4.14	3.88	4.16	4.05
Assayed peuNDF240om, % of DM[5]	6.35	6.07	8.60	8.00

[1]Amylase- and sodium sulfite-treated neutral detergent fiber, ash corrected.
[2]The 7-h starch degradability value was measured on the entire total mixed ration.
[3]Rumen fermentable starch: starch content multiplied by starch degradability.
[4]Physical effectiveness factor: measured by dry sieving with the 1.18-mm sieve (Mertens, 1997).
[5]Physically effective undegraded neutral detergent fiber after 240 h of in vitro fermentation, ash corrected. The uNDF240om from composited diet that was retained on ≥1.18-mm sieve. This value is sensitive to differences in uNDF240om distribution across dietary particle size fractions.

Table 3 summarizes the intake responses to the diets. There were no interactions between dietary peuNDF240 and RFS on DMI or intake of starch and uNDF240.There was no effect of either peuNDF240 or RFS on DMI in kg/d, but the higher peuNDF diets did slightly reduce DMI as a percentage of BW similarly for both RFS concentrations. The higher RFS diets reduced the intake of aNDFom which reflected the small differences between the diets in aNDFom content (Table 2). As expected, the higher RFS diets increased starch intake by approximately 18 to 20%. Likewise, the higher peuNDF240 diets increased uNDF240 intake by 9 to 14%; the content of dietary RFS also affected uNDF240 intake although the effect was very small.

Table 4 summarizes the milk and milk component responses to the diets. The higher peuNDF240 diets reduced milk yield by approximately 1.2 kg/d compared with the lower peuNDF240 diets. The daily yield of 3.5% fat-corrected milk (FCM) and ECM were both reduced by greater RFS content. Although there was no significant interaction between dietary peuNDF240 and RFS, the higher RFS reduced 3.5% FCM by 2.3 kg/d for the lower peuNDF diets versus only 0.7 kg/d for the higher peuNDF diets. It appears that the negative associative effect of RFS on FCM yield was more pronounced with the lower peuNDF240 diet. Again, it is important to remember that the uNDF240 and peuNDF240 (pef x uNDF240) values for all diets were at the lower range (approximately 7 and 4% of ration DM, respectively).

Table 3. Dry matter intake (DMI) and carbohydrate intake responses to experimental diets.

Variable	Diets				*P*-value[1]	
	Low peuNDF240		High peuNDF240			
	Low RFS	High RFS	Low RFS	High RFS	peuNDF	Starch
DMI, kg/d	29.7	29.4	29.4	29.2	0.27	0.40
DMI, % of BW/d	4.31	4.28	4.24	4.20	0.04	0.41
aNDFom[2] intake, kg/d	9.9	9.5	9.8	9.6	0.75	0.03
aNDFom intake, % of BW/d	1.44	1.39	1.42	1.37	0.37	0.03
Starch intake, kg/d	6.1	7.2	6.0	7.2	0.74	<0.0001
Starch intake, % of BW/d	0.88	1.06	0.87	1.04	0.35	<0.0001
uNDF240om intake, kg/d	2.25	2.16	2.45	2.40	<0.0001	0.008
uNDF240om intake, % of BW/d	0.322	0.315	0.354	0.345	<0.0001	0.0078

[1]There was no significant ($P > 0.10$) interaction between peuNDF240 and rumen fermentable starch.
[2]Amylase- and sodium sulfite-treated neutral detergent fiber, ash corrected.

Milk fat percentage was greater for the higher peuNDF240 than the lower peuNDF240 diets (Table 4). Similarly, milk fat percentage and daily output were depressed by the higher RFS versus the lower RFS diets. There was no significant interaction between peuNDF240 and RFS, although it is useful to note that numerically the highest milk fat percentage was for cows fed the higher peuNDF/lower RFS diet and the lowest milk fat percentage was with cows fed the lower peuNDF240/higher RFS diet. A negative associative effect existed between peuNDF240 and RFS that expressed itself in reduced milk fat. Overall, milk fat percentage was lower for all diets in this study compared with the typical milk fat percentage for the Institute dairy herd of approximately 4.0%. This general depression in milk fat likely reflected the lower uNDF240 and calculated peuNDF240 for all diets.

Table 4. Milk and milk component responses to experimental diets.

Variable	Diets				*P*-value[1]	
	Low peuNDF240		High peuNDF240			
	Low RFS	High RFS	Low RFS	High RFS	peuNDF	Starch
Milk, kg/d	53.1	52.0	51.2	51.5	0.01	0.35
3.5% FCM[2], kg/d	53.8	51.5	52.9	52.2	0.85	0.01
ECM[3], kg/d	53.4	51.5	52.5	51.9	0.56	0.02
Fat, %	3.59	3.48	3.74	3.60	0.05	0.06
Fat, kg/d	1.90	1.79	1.90	1.84	0.41	0.01
True protein, %	2.83	2.87	2.85	2.86	0.61	0.12
True protein, kg/d	1.50	1.48	1.45	1.47	0.02	0.94
Lactose (anhydrous), %	4.57	4.57	4.59	4.61	0.04	0.58
Lactose (anhydrous), kg/d	2.43	2.38	2.35	2.37	0.09	0.60
Urea nitrogen, mg/dL	12.0	10.1	12.4	10.5	0.08	<0.0001
De novo FA[4], g/100 g milk	0.80	0.76	0.81	0.80	0.15	0.26
Mixed origin FA, g/100 g milk	1.34	1.31	1.43	1.38	0.008	0.13
Preformed FA, g/100 g milk	1.31	1.26	1.34	1.29	0.17	0.02
De novo and mixed origin FA, g/100 milk	2.14	2.07	2.24	2.18	0.03	0.17
Unsaturation, double bonds/FA	0.288	0.294	0.281	0.280	0.005	0.43
3.5% FCM/DMI, kg/kg	1.81	1.75	1.81	1.79	0.41	0.06

[1]There was no significant ($P > 0.10$) interaction between peuNDF240 and rumen fermentable starch.
[2]Fat-corrected milk.
[3]Energy-corrected milk.
[4] Fatty acids.

Although milk fat yield was unaffected by peuNDF240 content, the yield of true protein was reduced slightly with higher peuNDF240 (Table 4). Milk urea nitrogen content tended to be increased by higher peuNDF240 and RFS substantially reduced milk urea nitrogen at either concentration of peuNDF240. These responses reflect greater efficiency of nitrogen use for cows fed the lower peuNDF240 and particularly the positive effect of moderately greater RFS on rumen nitrogen efficiency.

Mixed origin and mixed + de novo fatty acids were reduced by lower peuNDF240 diets versus higher peuNDF240. Likewise, the unsaturated fatty acids were increased for cows fed the low peuNDF240 diets. Numerically, cows fed the lower

peuNDF240/higher RFS diet that produced milk with the lowest milk fat percentage also had the least mixed + de novo fatty acids and highest unsaturated milk fatty acids. Overall, these changes in milk fatty acid composition track with the changes in milk fat percentage and indicate the onset of trans fatty acid-induced milk fat depression (Barbano et al., 2018). As a bottom line measure of herd performance, efficiency of FCM production (3.5% FCM/DMI) was lower for cows fed the higher RFS diets and it was least numerically for cows fed the lower peuNDF240/higher RFS diet. As a final "food for thought": in the post hoc analysis with CNCPS biology, it appeared that a RFS:uNDF240 ratio >2.8 might be a useful indicator for diets that have greater risk of milk fat depression. This idea requires further research to validate, but it seems to fit this data set.

Take Home Messages

As this research story unfolds, we plan to better define the interactions between RFS and fiber particle size and degradability to provide target values and benchmarks to use when formulating rations. To-date, take home messages of this research include:

- There is value in integrating forage particle size and uNDF240, and useful relationships exist between uNDF240 and peuNDF240 with DMI and ECM for high producing dairy cows.
- For corn silage-based diets, when uNDF240 exceeds 10 to 11% of ration DM, DMI may decrease; consider a finer chop length.
- uNDF240 less than 7% of ration DM may increase the risk of subacute rumen acidosis; maintain peNDF at least 19 to 20% of ration DM. Don't chop low uNDF240 forage too fine: cows still need effective NDF.
- peuNDF240 (pef x uNDF240) is a work-in-progress, but a range of 4.5 to 6% of ration DM seems to be a target for high producing cows fed corn silage-based diets.
- Associative effects among RFS, uNDF240, and peNDF are important. When peuNDF240 is approximately 4 to 6% of ration DM for corn silage-based diets (depending on how measured), and uNDF240 is <7.0% of ration DM, then negative effects of RFS on milk fat at only 19 to 20% of ration DM may occur.

- If dietary uNDF240 is not uniformly distributed across particle sizes, then direct measurement of uNDF240 in pef particle fraction may be a better approach. It will be critical not to confuse the two methods for measuring peuNDF240. Stay tuned.

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Dietary Sugars for Optimizing Rumen Function and Dairy Cow Performance

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Introduction

Typical US lactating dairy rations without supplemental sugars contain about 1.5 to 3% sugar. The use of more fermented forages and processed feeds has resulted in the removal of many sugars that would otherwise naturally be in the dairy cow diet. Sugars are water-soluble and include monosaccharides (glucose and fructose) as well as disaccharides (sucrose and lactose). Adding supplemental dietary sugar often reduces rumen ammonia, suggesting that rapidly digestible sugars help the rumen microbes capture and use nitrogen. Fiber digestion, microbial protein synthesis, energy absorption and rumen pH can increase with additional dietary sugars when balanced appropriately with dietary starch to positively impact dairy cow performance. Dietary factors such as physically effective fiber, level of starch, starch digestion rate, degradable proteins, and unsaturated fatty acids may affect cow response to supplemental sugars. Level of milk production and DIM may also influence responses to added dietary sugars.

Rumen Ammonia and Microbial Protein Synthesis

For efficient rumen microbial growth, availability of carbohydrate and protein is essential (Nocek and Russell, 1988). Work with continuous cultures of rumen microbes showed that microbial yield decreased curvilinearly from 34.2 to 10.3 g bacterial nitrogen per kg DM digested as the nonstructural carbohydrate / rumen degradable crude protein ratio widened from 1.9 to 8.9 (Hoover, 1987, Stokes et al., 1991). Aldrich et al. (1993) found the highest microbial protein yield (262 g/d) when a rapidly digestible protein source was fed with a rapidly digestible starch source and the lowest microbial protein yield (214 g/d) when a slowly digestible protein source was fed with a rapidly digestible starch source.

Additional dietary sugar almost always reduces rumen ammonia (Hoover and Miller-Webster, 1998), suggesting that sugars help the microbes capture and use dietary nitrogen. Dietary sugar above 7% has reduced ammonia concentrations (Broderick et al., 2008; Chibisa et al., 2015; McCormick et al., 2001), indicating improved in N utilization. Added dietary sugar has been shown to increase microbial protein synthesis (Chamberlain et al., 1993; Khalili and Huhtanen, 1991; Piwonka and Firkins, 1993), however, not consistently (Broderick et al., 2008; Sannes et al., 2002). Hall (2017) speculated that microbial protein yield from sugar fermentation would be increased in the presence of true proteins and peptides in the rumen. When given glucose as a substrate, rumen microbes preferred to use amino acids and peptides rather than urea (Hristov et. al., 2005).

Rumen pH

Sub-clinical rumen acidosis (SARA) occurs when the pH of the cow's rumen drops below 5.8. Excessive production of rumen lactic acid, primarily from the fermentation of starch, reduces pH. At low rumen pH, hydrogen ions leak inside the microbes. To maintain near neutral pH within their cells, the microbes must expend energy to expel hydrogen ions, resulting in less energy available for growth (Strobel and Russell, 1986). Those microbes that ferment fiber are most negatively affected by rumen acidity (Russell and Dombrowski, 1980).

Supplemental sugars may improve rumen pH via a few different mechanisms. First, lower rumen propionate would be expected if sugar was substituted for dietary starch (Bannink et al., 2006). Second, if sugars improve efficiency of microbial protein production and are incorporated into the microbial mass, less rumen degraded OM would be converted into fermentation acids. Increasing microbial efficiency from 20 to 30 g microbial N per kg of rumen degraded OM should result in a 12.5% reduction in fermentation acids (Allen, 1997; Penner et al., 2009). Further, because sugars are rapidly available, they are more apt to be converted into the storage polysaccharide, glycogen, by rumen bacteria and protozoa (Hall, 2017), slowing fermentation to control rumen acidity.

Dietary sugar often increases the molar proportion of butyrate (Chibisa et al., 2015; DeFrain et al., 2004; Oba et al., 2015; Penner et al., 2011; Sun et al., 2015; Vallimont et al., 2004). Butyrate generates only one H^+ while propionate and acetate generate 2 H^+. Butyrate stimulates the rumen epithelial cells, increasing VFA absorption from the rumen (Oba et al., 2015). In continuous culture, Vallimont et al. (2004) linearly increased butyrate from 12.2 to 13.8, 13.7, and 14.2 mol/100 mol when 0, 2.5, 5.0, and 7.5% sucrose was supplemented. Higher rumen butyrate concentrations may improve both rumen epithelial absorption of acids and glucose transport, to moderate rumen pH (Oba et al., 2015; Penner et al., 2011).

Penner et al. (2009) replaced cracked corn grain with sucrose to produce diets containing either 2.8 or 5.7% sugar. The high sugar diets resulted in a higher daily minimum rumen pH (5.61 vs. 5.42) as well as a higher mean rumen pH (6.30 vs. 6.17). Postpartum transition cows fed 8.4% vs. 4.7% dietary sugar tended to have higher nadir (5.62 vs. 5.42), mean (6.21 vs. 6.06) and maximum rumen pH (6.83 vs. 6.65) (Penner and Oba, 2009). Cows fed diets designed for milk fat depression (> 33% starch) had significantly higher rumen pH (5.87 vs. 5.73) when 5% of the diet DM from corn was replaced with molasses (Martel et al., 2011).

Milk Fat

A common effect of sugar supplementation is an increase in milk fat percentage and/or yield. This can be explained by a number of mechanisms. First, as previously discussed, sugars increase the molar proportion of butyrate and butyrate is used for milk fat synthesis. Second, if sugars moderate rumen pH as previously described, one would expect a positive relationship of rumen pH on milk fat percentage (Allen, 1997). Finally, sugars impact fatty acid biohydrogenation. When Sun et al. (2015) replaced corn starch with 3, 6, or 9% sucrose, *Butyrivibrio fibrisolvens* numbers increased. *Butyrivibrio fibrisolvens* produces both butyrate and CLA cis-9, trans-11 which is part of the normal fatty acid biohydrogenation pathway. At the same time, numbers of *Megasphaera elsdenii* were decreased thus inhibiting production of the trans-10 isomer of the 18:1 fatty acid implicated in milk fat depression.

When Broderick et al. (2008) replaced starch with 2.5, 5, and 7.5% sucrose, milk fat yield increased from 1.47 to 1.53, 1.65, and 1.62 kg/cow/day, respectively with the effect at 5% sucrose being statistically significant ($P<0.05$). Milk fat percentage changed from 3.81 to 3.80, 4.08, and 4.16%, with the positive effects at 5 and 7.5% sucrose being statistically significant ($P<0.05$). Postpartum transition cows fed 8.4% vs. 4.7% dietary sugar tended to have higher milk fat yield (1.44 vs. 1.35 kg/d) (Penner and Oba, 2009). Cows fed high starch diets (> 46% NFC) designed for milk fat depression responded with higher milk fat concentrations (3.01 vs. 2.61%), specifically from short- and medium-chain fatty acids, when 5% of the diet DM from corn was replaced with molasses (Martel et al., 2011).

Fiber Digestion

In a few studies, added dietary sugars have improved fiber digestion. Firkins (2011) suggested that sugar fermenting bacteria may provide growth factors and improve the environment for fluid-associated fiber-digesting bacteria in the rumen. Improvements in rumen pH as a result of sugar supplementation should also positively impact fiber digestion. Broderick et al. (2008) showed a positive quadratic effect on fiber digestion when they replaced corn starch with sugar (2.5, 5, and 7.5% sucrose) in a 60% forage diet. Both ADF and NDF digestion were highest with the addition of 5% sucrose (7.1% total dietary sugar). When Broderick and Radloff (2004, Trial 2) used liquid molasses to replace high moisture corn in a 60% forage diet to increase dietary sugar (2.6, 4.9, 7.4 and 10% of diet DM), fiber digestion was significantly higher with the 7.4% sugar diet.

Intake and Production

Supplemental sugars have generated variable intake and milk production results in published studies. Although Broderick et al. (2008) increased DM intake and yield of milk fat with added dietary sugar, effects on milk and fat-corrected milk yield were not significant. Postpartum transition cows fed 8.4% vs. 4.7% dietary sugar had higher DM intake (18.3 vs. 17.2 kg/d) but milk yield was not affected, averaging 33.7 kg/d (Penner and Oba, 2009). Adding a liquid molasses product to a TMR at a rate of 4.1% increased

dietary sugar from 4 to 5.4% to reduce TMR sorting as well as improve DM intake (27.7 vs. 29.1 kg/d) and 4% FCM yield (39.7 vs. 42.8 kg/d) (DeVries and Gill, 2012).

When Broderick and Radloff (2004, Trial 1) incrementally replaced high-moisture corn with dried molasses (2.6, 4.2, 5.6 and 7.2% dietary sugar), there was a positive quadratic response in milk fat content, yield of fat, and FCM with maximum responses occurring at 4.2 to 5.6% dietary sugar. Dry matter intake increased by 1 kg/cow/d (26.3 vs. 25.3 kg/cow/d) with 5.6% vs. 2.6% dietary sugar.

Replacing corn grain with sugar to reduce dietary starch from 32 to 27% and increase dietary sugar from 4.5 to 9% resulted in higher DM intake (27.5 vs. 26.2 kg/d), higher ECM (39.6 vs. 38 kg/d) and higher milk CP yield (1.31 vs. 1.26 kg/d) (Gao and Oba, 2016).

Predicting Dairy Cattle Response to Added Dietary Sugars

The impact of supplemental dietary sugar on dairy cow responses was determined using an 85 observation dataset from published research, while accounting for the effects of other diet nutrients and cow factors including DIM and production level (de Ondarza et al., 2017). Sugar sources included molasses, whey, and dry sugar (sucrose or lactose). Dietary forage NDF was 17.4 to 29.5%, typical of commercial US dairy diets. Diet nutrient profiles were determined by entering diet and feed analysis data from each experiment into an advanced nutrition model (CNCPS 6.1 with NDS platform, RUM&N Sas, Italy). Mixed model linear regression analysis was conducted using the Fit Model function of JMP statistical software (SAS Inst. Inc, Cary, NC). The model fit used treatment category (control, 1.5-3%, 3-5%, vs. 5-7% added dietary sugar (% of diet DM)), DIM category (< 150 or > 150 DIM) within treatment, control milk yield category (> 33 or < 33 kg/d) within treatment, and the following nutrient variables (% of diet DM) as continuous variables: starch, soluble fiber, forage NDF, ammonia, RDP, and protein B_2 (insoluble in boiling neutral detergent but soluble in boiling acid detergent solution). Number of cows per treatment was included as a weighting factor and experiment was included as a random effect. A description of the dataset including number of treatment means reported for each study, number of cows per treatment, mean DIM, and control 3.5% FCM (kg/d) for each study is presented in Table 1. Mean performance and diet characteristics are reported in Table 2. Days in milk ranged from 14 to 252. Fat-corrected milk yield in control cows ranged from 18 to 45 kg/cow/d.

Table 1. Published research studies used to determine the effect of additional dietary sugar on dairy cattle performance (adapted from de Ondarza et al., 2017).

Experiment	Number of Treatment Means	Number of Cows per Treatment	Mean DIM	Control 3.5% FCM, kg/cow/d
Baurhoo and Mustafa, 2014	3	12	129	38
Broderick et al., 2008	3	12	112	41
Broderick & Radloff, 2004 #1	3	12	167	41
Broderick & Radloff, 2004 #2	4	12	120	45
Cherney et al., 2003	4	20	98	38
Chibisa, 2013	4	8	165	41
De Frain et al., 2004	3	12	252	25
De Vries and Gill, 2012	2	12	109	43
Eastridge et al., 2011 #1	4	5	219	35
Eastridge et al., 2011 #2	4	12	109	41
Firkins et al., 2008 #1	4	10	81	36
Firkins et al., 2008 #2	5	10	81	34
Firkins et al., 2008 #3	4	12	112	38
Golombeski et al., 2006	4	12	173	30
Hall et al., 2010	4	18	114	40
Hindrichsen et al., 2006	3	6	223	18
Maiga et al., 1995	3	10	74	35
McCormick et al., 2001	4	8	100	38
Nombekela & Murphy, 1995	2	16	42	28
Oelker et al., 2009	5	7	202	36
Penner et al., 2009	4	8	205	24
Penner and Oba, 2009	2	25	14	37
Sannes et al., 2002	4	16	149	36
Siverson et al., 2014	4	40	238	31
Vargas-Rodriguez et al., 2014	2	48	157	35

Additional dietary sugar increased yield of milk, 3.5% FCM, and milk true protein (P<0.05) (Table 3). Milk yield was 31.91 kg/cow/d with no added sugar and increased (P=0.03) to 33.33 and 33.02 kg/cow/d with 3-5% and 5-7% added dietary sugar (% of diet DM), respectively. Likewise, 3.5% FCM increased (P=0.04) from 32.35 to 33.80 kg/cow/d with 5-7% added dietary sugar (% of diet DM). Milk true protein yield increased (P=0.05) from 0.98 kg/cow/d without supplemental sugar to 1.05 kg/cow/d with 5-7% added dietary sugar (% of diet DM). Increased milk true protein yield suggests a possible increase in rumen microbial protein synthesis with dietary sugar addition as observed by Chamberlain et al. (1993) and Khalili and Huhtanen (1991). Unlike the results of others (Broderick et al., 2008; Firkins et al., 2008), DM intake and milk fat percentage were not significantly increased (P>0.20) with additional sugar across these studies. Milk urea nitrogen was numerically lower with increasing supplemental sugar but this change was not statistically significant (P>0.20). Feed efficiency was not significantly impacted by sugar addition (P=0.13).

Table 2. Mean performance characteristics and diet nutrient parameters of published research studies used to determine the effect of additional dietary sugar on dairy cattle performance (adapted from de Ondarza et al., 2017).

	Mean	SD
DIM	142	58
DMI, kg	23.48	2.83
Milk, kg	35.30	5.79
Milk true protein, %	3.06	0.25
Milk true protein, kg	1.07	0.16
Milk fat, %	3.61	0.37
Milk fat, kg	1.27	0.22
3.5% FCM, kg	35.83	5.80
MUN, mg/dl	14.06	2.80
% Forage	50.85	6.45
CP, %DM	17.36	1.28
Ammonia, %DM	0.75	0.58
Protein B_2, %DM[a]	1.41	0.48
RDP, %DM	10.74	1.11
NDF, %DM	32.16	3.93
Forage NDF, %DM	22.89	2.85
Sugar, %DM	5.57	2.04
Starch, %DM	23.68	4.99
Soluble Fiber, %DM	6.44	1.93

[a] Protein that is insoluble in boiling neutral detergent but soluble in boiling acid detergent solution

Cows producing > 33 kg milk/d had greater responses to added dietary sugar (P<0.0001). Cows producing > 33 kg/d of milk produced 2.14 kg/d more 3.5% FCM with 5-7% added dietary sugar (% of diet DM) (37.78 vs. 39.92 kg/d). However, cows producing < 33 kg/d only responded with 0.77 kg/d more 3.5% FCM (26.91 vs. 27.68 kg/d) (Figure 1). Similar differences were observed with milk true protein yield (P<0.0001), increasing by 0.09 vs. 0.05 kg/cow/d with 5-7% added dietary sugar (% of diet DM) for higher vs. lower producing cows (Figure 2).

Ruminal VFA concentrations were impacted by dietary sugar addition (Table 3). Level of added dietary sugar tended (P<0.10) to affect rumen butyrate concentrations, increasing with 5-7% added dietary sugar (Table 3). Acetate and propionate (mM) decreased (P<0.05) with added dietary sugar.

Table 3. The effect of additional dietary sugar category (control, 1.5-3%, 3-5%, vs. 5-7% added dietary sugar) on DMI, milk yield, 3.5% FCM, milk components, feed efficiency, and ruminal VFA concentrations (adapted from de Ondarza et al., 2017).

	Added Dietary Sugar (%DM)								
	Control		1.5-3%		3-5%		5-7%		*P*-Value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
DMI, kg/d	22.16	0.52	22.40	0.51	21.99	0.57	22.92	0.82	0.22
Milk, kg/d	31.91	0.65	32.90	0.65	33.33	0.73	33.02	1.11	0.03
FCM, kg/d	32.35	0.56	33.37	0.57	33.72	0.66	33.80	1.11	0.04
TP, %	3.14	0.04	3.11	0.04	3.13	0.05	3.16	0.07	0.42
TP, kg	0.98	0.02	1.01	0.02	1.04	0.02	1.05	0.04	0.05
MF, %	3.64	0.08	3.61	0.08	3.56	0.08	3.55	0.12	0.65
MF, kg	1.14	0.02	1.18	0.02	1.19	0.03	1.20	0.05	0.16
MUN mg/dl	14.19	0.43	13.80	0.45	13.56	0.52	12.58	0.88	0.27
FE[a]	1.46	0.03	1.48	0.03	1.52	0.03	1.46	0.05	0.13
Ac, mM	68.65	3.37	64.90	3.25	60.35	3.48	60.17	4.17	0.02
Pr, mM	22.92	1.04	22.22	1.03	21.28	1.17	17.86	1.50	0.03
Bu, mM	12.81	0.65	11.79	0.66	11.68	0.77	13.19	0.99	0.06

[a] kg 3.5% FCM/kg DMI

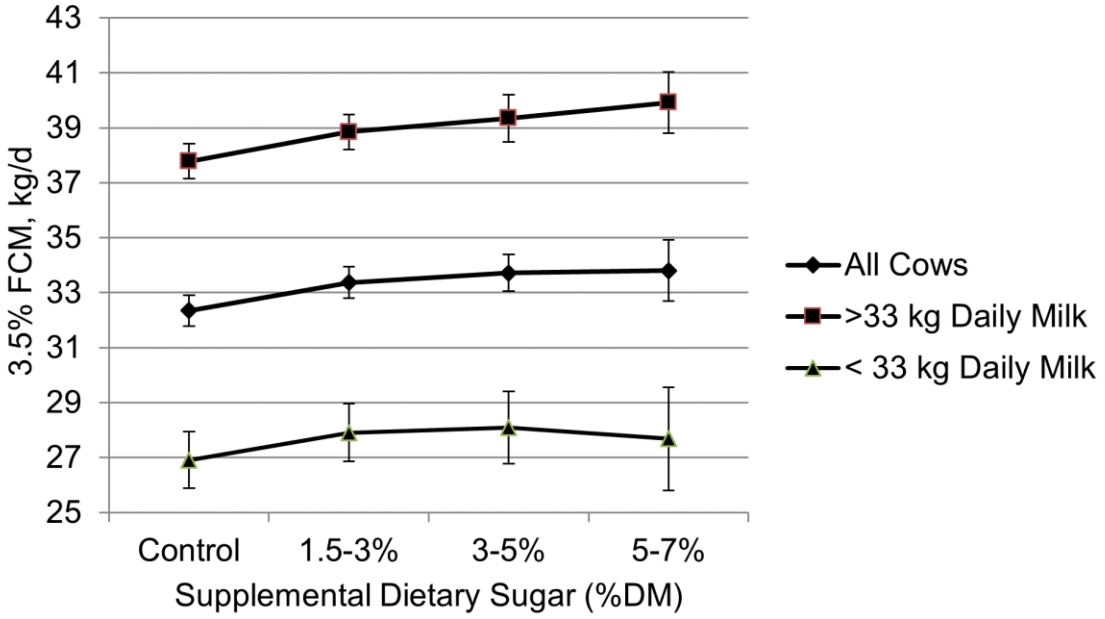

Figure 1. Effect of additional dietary sugar on 3.5% FCM by production level in published research studies (all cows vs. > 33 kg (high yield cows) vs. < 33 kg (low yield cows) (adapted from de Ondarza et al., 2017).

Table 4. Nutrient parameter estimates for variables tending (P<0.10) to effect milk and milk component production in published research studies used to determine the effect of additional dietary sugar on dairy cattle performance (adapted from de Ondarza et al., 2017).

	Milk, kg	Milk TP, %	Milk TP, kg	Milk fat, %	Milk fat, kg	MUN, mg/dl
Starch, %DM	+0.31	---	+0.02	-0.02	+0.01	-0.20
Soluble Fiber, %DM	---	---	---	---	+0.02	-0.67
RDP, %DM	---	---	---	---	---	+1.30
Protein B_2, %DM[a]	+1.78	---	---	---	---	+1.78

[a] Protein that is insoluble in boiling neutral detergent but soluble in boiling acid detergent solution

Nutrient parameters that tended (P<0.10) to affect milk and milk component production are recorded in Table 4. As expected, increased dietary starch improved milk and milk true protein yield while tending to decrease milk fat percentage and MUN (mg/dl). Soluble fiber reduced MUN (mg/dl). Increases in RDP increased MUN (mg/dl) while increases in protein B_2 tended to increase milk yield and MUN (mg/dl).

Non-linear analysis indicated that to optimize 3.5% FCM yield response when feeding additional sugars, a low to moderate starch diet should be fed (22 to 27% of diet DM) in combination with a moderate to high soluble fiber content (6 to 8.5% of diet DM) while 6.75 to 8% DM of dietary sugar was ideal (de Ondarza et al., 2017).

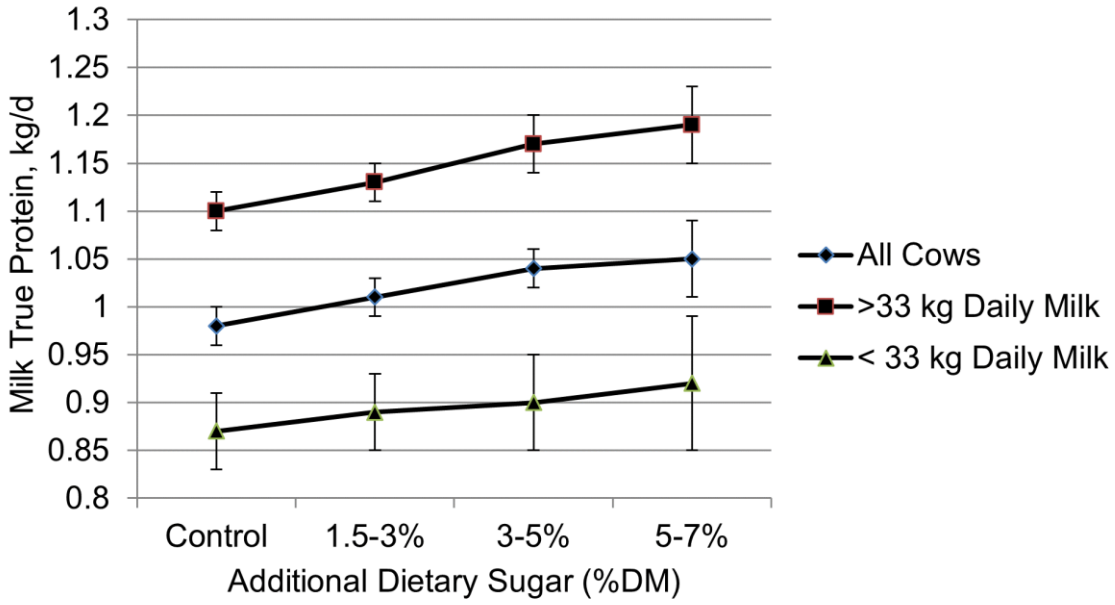

Figure 2. Effect of additional dietary sugar on milk true protein yield (kg/d) by production level in published research studies (all cows vs. > 33 kg (high yield cows) vs. < 33 kg (low yield cows) (adapted from de Ondarza et al., 2017).

Practical Applications

Consider supplementing sugar in lactating dairy diets to achieve 6 to 8% diet sugar for optimum rumen function and performance. Generally, 0.7 to 1.0 kg/cow/d of supplemental sugar would be needed to achieve 6 to 8% total sugar in typical US diets. Higher producing cows would be expected to have more positive responses to added dietary sugar. Liquid sugar sources have the added benefit of reducing TMR sorting.

Recognize the interactions between sugar, starch, soluble fiber, and rumen degradable protein. Research and field experience suggest the following optimal nutrient ranges (%DM): starch at 22 to 27%, soluble fiber at 6 to 8%, and RDP at 10 to 11%. Further, consider the impact of starch and protein degradation rates on responses to supplemental sugars. Sugars would be expected to have a more positive effect with a diet containing a lower percentage of rapidly digestible starch. Consider increasing soluble protein, using milk urea nitrogen (MUN) levels as a guide.

Future research to characterize and understand the effects of dietary sugars by type (glucose, sucrose, fructose, lactose, etc.) as well as to define multiple starch pools based on digestion rate and understand their impact on dietary sugar optimization would be helpful.

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Daily Rollercoaster of Blood and Milk Metrics

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Introduction

Dairy cows often enter a state of energy deficit in early lactation, leading to an increase in plasma concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB). Currently, diagnosis of excessive energy deficit is done on farms using handheld blood BHB meters. However, this process is laborious and can become costly when used as a whole-herd screening method. Several studies have investigated the use of Fourier transform mid-infrared (FTIR) estimates to predict excessive energy deficit through milk (Denis-Robichaud et al., 2014; Santschi et al., 2016; Bach et al., 2019), but many of these studies relied on a single, test-day DHIA milk sample with no knowledge of actual blood NEFA or BHB concentrations. Here we present our investigation of the diurnal variation in plasma NEFA and BHB as well as FTIR estimates of milk BHB and predicted blood NEFA, with particular interest in differences between groups of cows that were hyperketonemic or non-hyperketonemic. This information will improve knowledge and usability of on-farm testing results and promote discussion of the benefits of routine milk testing and analysis.

Study Design & Results

We collected blood samples every 2 h for 5 consecutive days from 28 multiparous Holstein cows that were between 3 and 9 days in milk. Cows were housed in a tie-stall facility and offered free choice access to water and a TMR that was delivered once a day at 0900 h. Blood samples were analyzed for BHB and NEFA concentrations, and cows were classified into hyperketonemia groups based on their average daily BHB concentration. If a cow's average daily BHB was ≥1.2 mmol/L for ≥3 study days, she was assigned to the hyperketonemia group (n=13). Alternatively, if her average daily BHB was ≥1.2 mmol/L for ≤2 study days, she was assigned to the non-hyperketonemia group (n=15).

Blood Results

We saw the lowest concentrations of BHB just prior to feeding, at 0700 h, with a steady rise following feed delivery (Figure 1A). Not surprisingly, BHB was higher in the hyperketonemic cows than the non-hyperketonemic cows (Figure 1B). Unlike BHB however, we saw a peak in NEFA just prior to feeding at 0700 h, with concentrations falling quickly after feed delivery (Figure 1C). The hyperketonemic cows had greater concentrations of NEFA than the non-hyperketonemic cows (Figure 1D).

To understand the effect of hyperketonemic on the daily fluctuations of BHB and NEFA, we calculated the difference between the daily maximum and minimum concentrations for each metabolite by hyperketonemia group. The hyperketonemic cows experienced a nearly two-fold greater difference between daily maximum and minimum BHB concentration as compared to the non-hyperketonemic cows. Interestingly, the difference between daily maximum and minimum concentrations of NEFA were relatively similar for both the hyperketonemic and non-hyperketonemic cows.

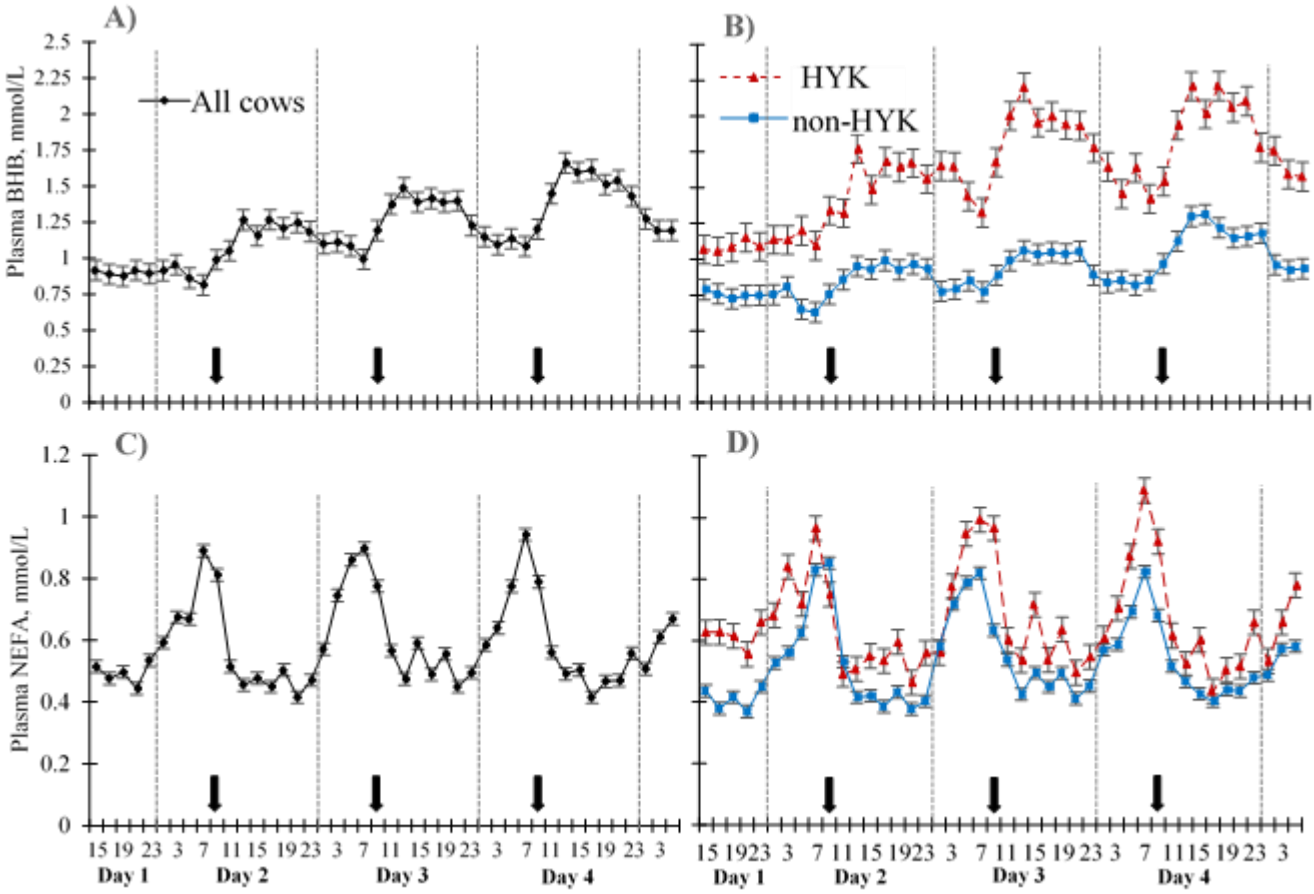

Figure 1. Concentrations of BHB and NEFA from multiparous Holstein cows classified as hyperketonemic (HYK; average daily mean BHB ≥1.2 mmol/L for ≥3 study days, red dashed line) or non-hyperketonemic (non-HYK; average daily mean BHB ≥1.2 mmol/L for ≤2 study days, solid blue line). Black arrows indicate time of feed delivery (Adapted from Seely et al., *Journal Dairy Science*, In Press).

Milk Results

We saw similar diurnal findings with mid-FTIR milk predicted metabolites, however with a general lag in peak or nadir concentrations than blood. The lowest milk BHB and milk predicted blood NEFA concentrations were at the morning milking just prior to feeding (Figure 2A, 2C). As for blood, predicted milk BHB and milk predicted blood NEFA were higher in hyperketonemic than non-hyperketonemic cows (Figure 2B, 2D). However,

unlike blood, difference in milk BHB between hyperketonemic groups was more consistent, and the pattern of diurnal variation in milk predicted blood NEFA never overlapped between the two groups.

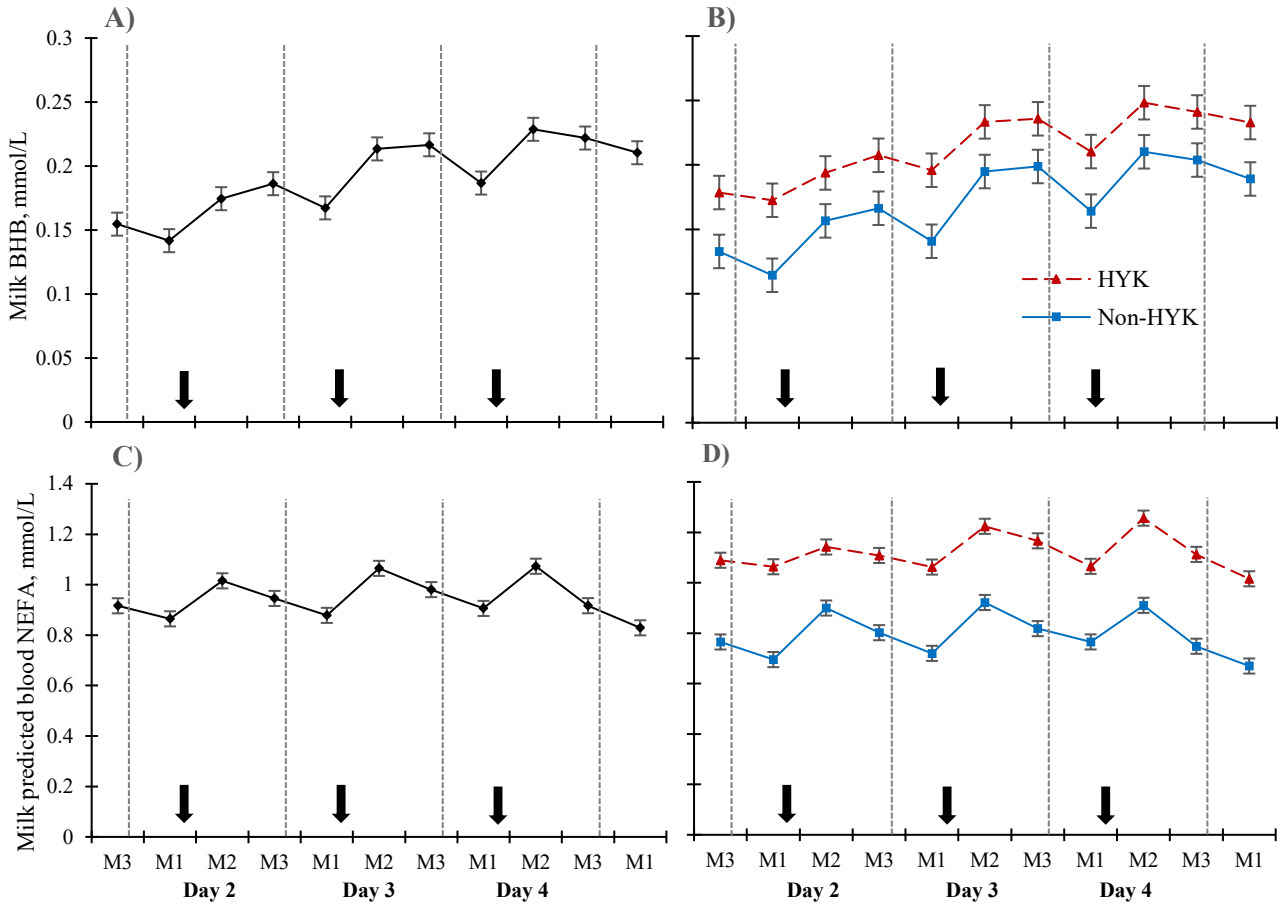

Figure 2. Concentrations of mid-FTIR milk predicted metabolites (milk BHB and milk predicted blood NEFA) from multiparous Holstein cows classified as hyperketonemic (HYK; average daily mean BHB ≥1.2 mmol/L for ≥3 study days, red dashed line) or non-hyperketonemic (non-HYK; average daily mean BHB ≥1.2 mmol/L for ≤2 study days, solid blue line). Black arrows indicate time of feed delivery; M1 = morning milking, M2 = afternoon milking, M3 = evening milking (Unpublished data from Seely, McArt, and Barbano).

Cows were milked 3 times per day. Therefore, each milk sample theoretically represents the average of what happened in the blood for the 8 h period prior to milking. We averaged the 2 h blood testing data over each 8 h period, prior to each milking, to achieve better correspondence of the time period for milk and blood results. Milk predicted blood NEFA, milk BHB, and milk fatty acids were measured at each milking using a mid-FTIR milk analysis (Delta FTA, Perkin-Elmer Corp., Drachten, The Netherlands). The data for milk predicted blood NEFA and blood NEFA over consecutive milkings for the hyper and non-hyperketonemic groups of cows are shown in Figures 3 and 4. Both the blood NEFA and milk estimated blood NEFA cycled during each 24 h period with a slight

lag in timing of the cycling. Both the blood NEFA and the milk estimated blood NEFA clearly separated the two groups of cows.

The concentration of milk BHB was also measured by infrared milk analysis and concentration in milk also cycled (data not shown).

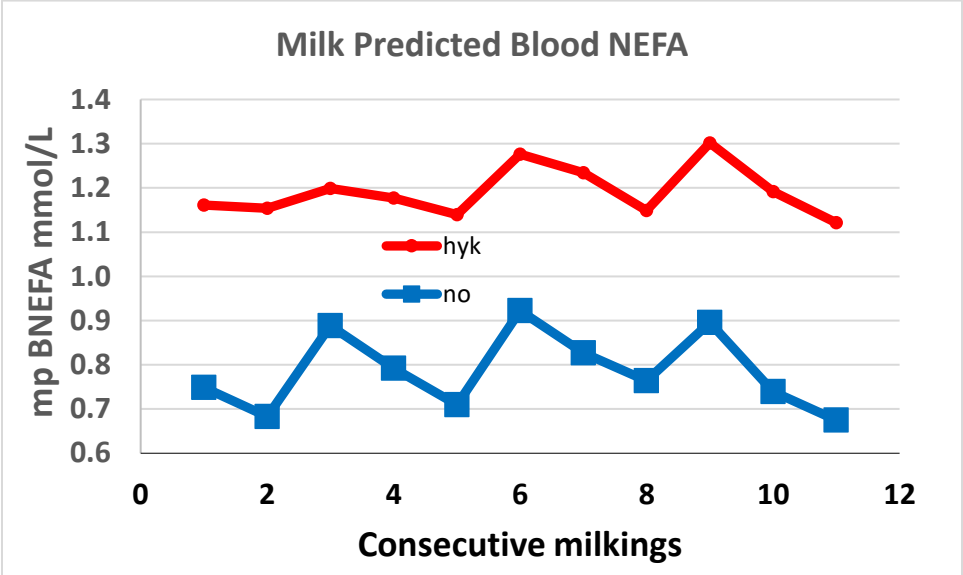

Figure 3. Milk estimated blood NEFA over consecutive milkings.

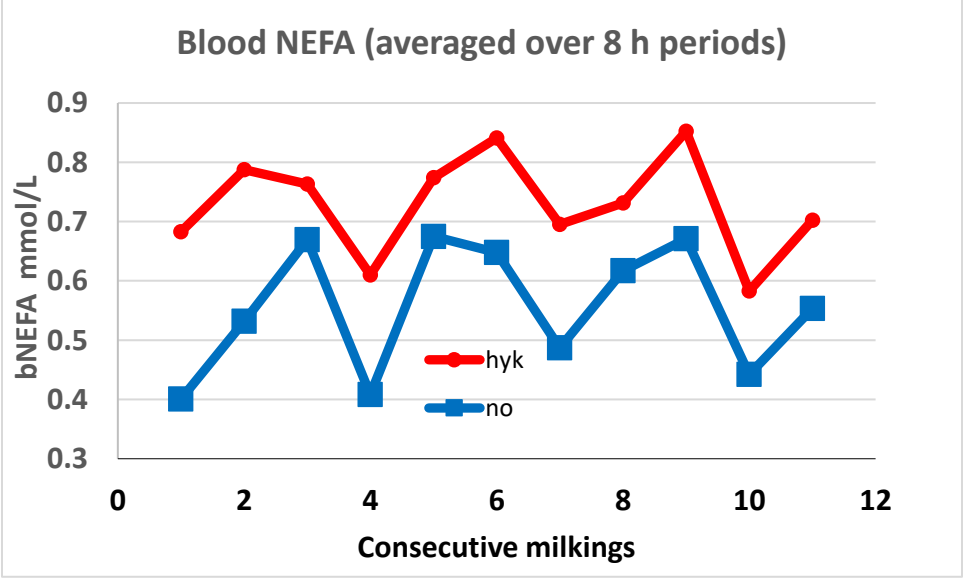

Figure 4. Blood NEFA (2 h samples averaged across the 8 h period prior to milking) plotted as a function of consecutive milkings.

Milk fatty acids (denovo, mixed origin, and preformed) were also measured at every milking by mid-infrared milk analysis (Figures 5, 6, and 7), as described by Wojciechowski et al. 2016 and Woolpert et al. 2016. The comparison of relative concentration for the 3 different groups of milk fatty acids between hyper and non-hyperketonemic groups of cows was clearly separated for all three milk fatty acid metrics. The non-hyperketonemic cows had higher relative concentrations of de novo and mixed origin milk fatty acids and lower performed milk fatty acids than the hyperketonemic cows. The cycle phasing of the relative concentrations of the de novo and mixed origin fatty acids had the opposite phasing when compared with the phasing of the preformed milk fatty acid cycling. Cycling of the relative concentration of milk fatty acid groups (Figures 5, 6, 7) was related to cycling of blood NEFA and the cycling of the fatty acid groups was consistent with the milk estimated blood NEFA cycling (Figure 3). When milk estimated blood NEFA was at a maximum of a cycle, preformed fatty acids were also at the maximum. Immediately before the cows were given fresh feed, milk estimated blood NEFA and relative concentration of milk preformed fatty acids were at a maximum and the de novo and mixed origin fatty acids were at a minimum.

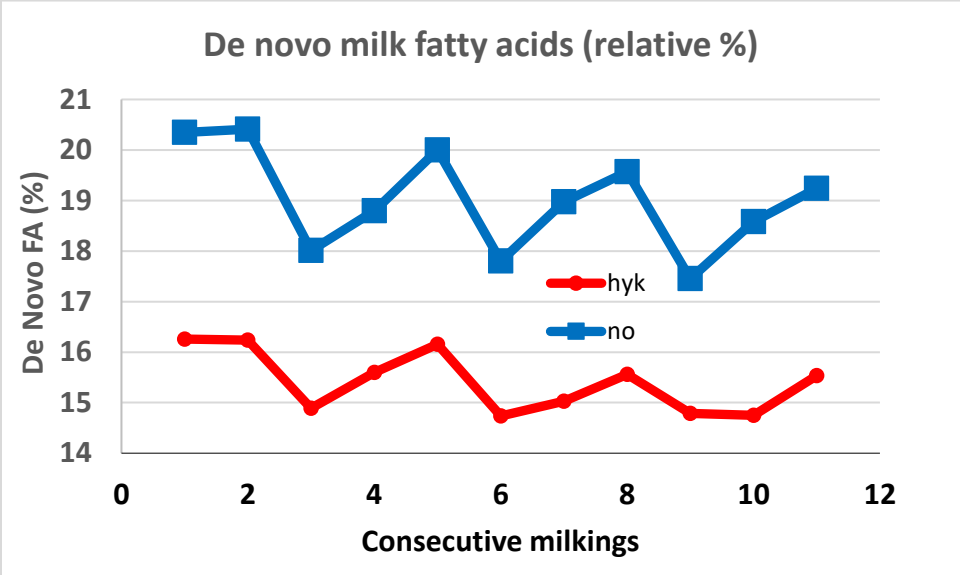

Figure 5. De novo milk fatty acids (relative %) measured by mid-infrared milk analysis. Fresh feed offered immediately after milking 3, 6, and 9.

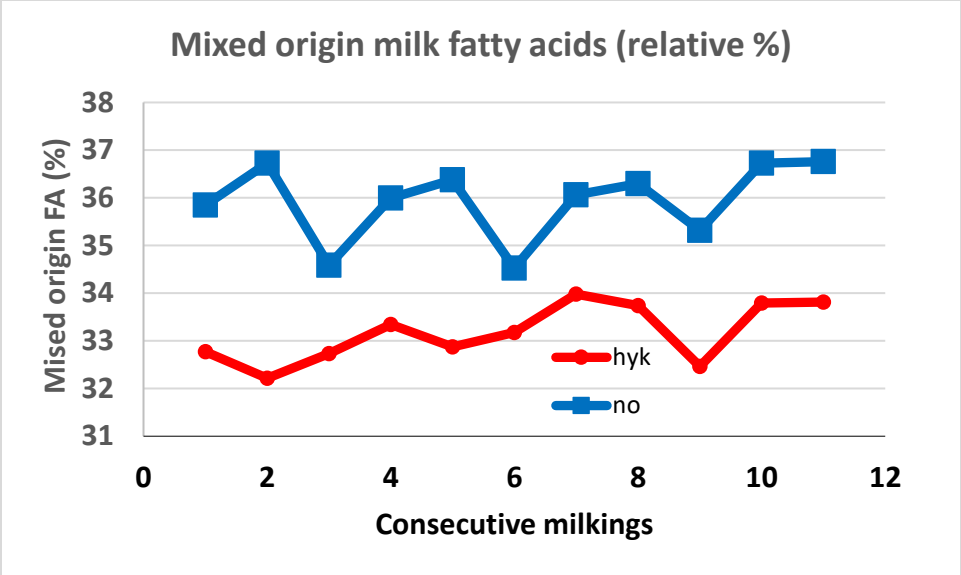

Figure 6. Mixed origin milk fatty acids (relative %) measured by mid-infrared milk analysis. Fresh feed offered immediately after milking 3, 6, and 9.

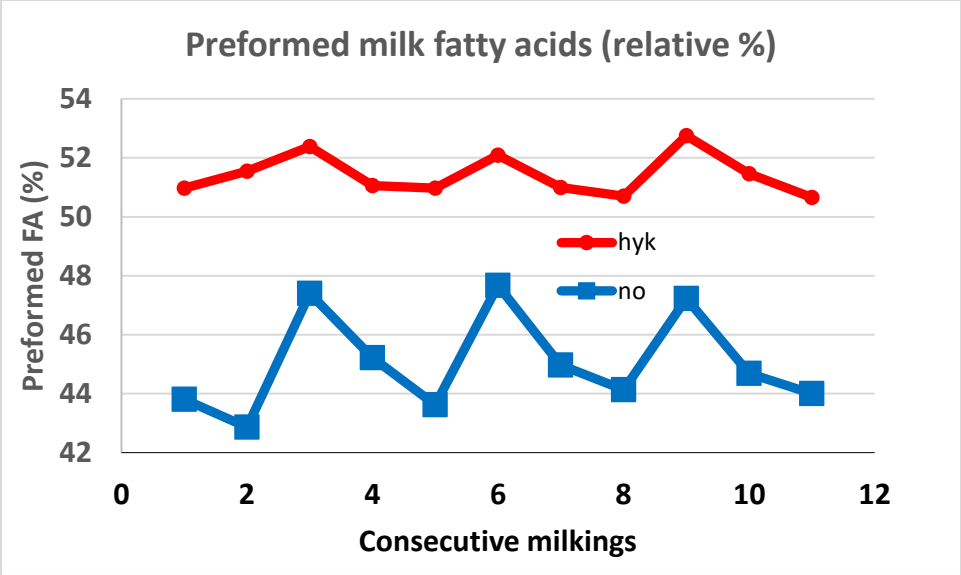

Figure 7. Preformed milk fatty acids (relative %) measured by mid-infrared milk analysis. Fresh feed offered immediately after milking 3, 6, and 9.

We hypothesize that the differences between peak and nadir blood and milk metabolites are due to milk having a higher correlation with an 8-hour average of blood metabolite concentrations rather than a single blood sample. This makes biological sense and also supports the idea that milk analysis might be an improved method of representing a cow's overall energy status than a single snapshot in time as currently provided with blood sampling.

Summary

We see clear and consistent diurnal patterns in plasma BHB and NEFA as well as FTIR estimates of milk BHB and milk predicted blood NEFA. The amplitude in change between daily maximum and minimum plasma metabolites and milk constituents were also affected by hyperketonemia status. Interestingly, these diurnal differences were much more predictable when analyzing milk, with a greater ability to separate hyperketonemic from non-hyperketonemic cows. Our results support the use of FTIR estimates of milk BHB and milk predicted blood NEFA as a tool in diagnosing HYK, however time relative to feeding should be considered when analyzing results. Milk fatty acid metrics on a relative basis may also be useful to separate hyperketonemic from non-hyperketonemic cows. In particular, these results support the use of milk monitoring and measurement to detect alterations in early lactation health of dairy cows.

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From Membrane Biophysics to the Farm: Applications of Fatty Acid and Monoglyceride Chemistry to Animal Health

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Introduction

Antibiotics have long been used in the livestock industry for therapeutic purposes to prevent severe clinical disease and death [Boyd et al, 2019]. Antibiotics work against bacteria and different classes of antibiotics have distinct targeting spectrums and potencies [Gustafson and Bowen, 1997]. Certain antibiotics have also been and continue to be used for treating subclinical diseases [Dibner and Richards, 2005], and successful intervention can improve livestock growth and feed efficiency [Cromwell, 2002]. Mechanistically, antibiotics can affect bacterial cell integrity and reproduction, and thus exert growth-promoting effects by modulating the composition of bacterial populations within the gastrointestinal tract. They can also reduce bacterial pathogen levels throughout the body to prevent disease.

Despite these important capabilities, there are mounting concerns that antibiotic usage in food animals is contributing to the global problem of antibiotic-resistant bacteria across human and animal populations [Barton, 2014]. These concerns have led to calls to significantly reduce or stop the use of antibiotics in animals that are also used in human medicine, particularly in cases where they are used at sub-therapeutic levels to promote growth [Marshall and Levy, 2011]. Legislative bans on growth-promoting antibiotics have been passed in different parts of the world, such as the European Union [Cogliani, et al, 2011], while other regulatory actions, such as the revised Veterinary Feed Directive in the United States, are encouraging more judicious use of human medically relevant, therapeutic antibiotics in food animal production, including halting sub-therapeutic use of such antibiotics [Schulz, et al, 2017].

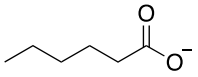
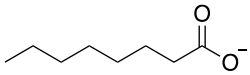
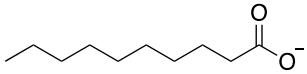
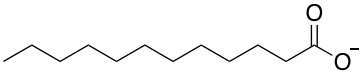
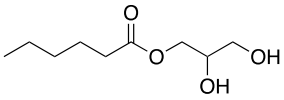
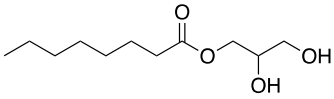
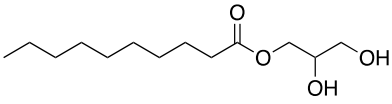
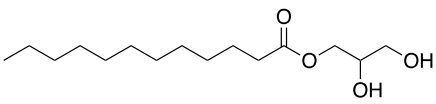
Concerns and legislative actions around antibiotic use in food animals are leading to an increase in the search for tools to counter pathogens. The increasing threat of viral pathogens that are foreign to North America, has also prompted the search for a means to directly destroy viruses. A recent addition to vaccines, therapeutic drugs, and immune enhancers is medium-chain fatty acids (MCFAs), especially saturated MCFAs with 6-12 carbon-long chains. They have proven benefits as feed additives by improving animal health, performance, and nutrient digestibility [Baltić, et al 2017]. Importantly, MCFAs and their monoglyceride derivatives (MCMGs) exhibit both antiviral and antibacterial activity [Thormar and Hilmarsson, 2997; Messens et al, 2010]. The combined health-promoting and pathogen-mitigating functions of MCFAs and MCMGs are particularly significant in light of the ongoing challenges of porcine epidemic diarrhea virus (PEDv), porcine reproductive and respiratory syndrome virus (PRRSv) and with the emerging threat of

African swine fever virus (ASFv) in pigs, as well as with the ongoing health issues of bovine coronavirus, bovine herpes virus 1 (BHV-1) responsible for Infectious bovine rhinotracheitis and bovine respiratory syncytial virus in calves. Formaldehyde is one common mitigant that is used to reduce the risk of these viruses and bacterial pathogens in feed, but it is under increasing regulatory pressure due to the hazards associated with its use and residue in feed. Over the past few years, there has been progress in understanding the mode of action of MCFAs and MCMGs as antivirals and antimicrobials and there is promise in both monogastric and ruminant production for their potential to replace antibiotics while improving animal health and productivity. In addition to multifunctional inhibitory activity against both viruses and bacteria, this class of antibiotic replacement candidates is additionally distinguished from the myriad of other candidates by an ability to be absorbed and transit to systemic sites of viral infection (Jackman et al., 2020). Replacement strategies must include this facet.

Physical Properties of MCFAs and MCMGs

MCFAs and MCMGs are single-chain lipid amphiphiles. An overview of the basic physical properties of important MCFAs and MCMGs is presented in Table 1.

Table 1. Overview of MCFAs and MCMGs

	Compound Name (Molecular Formula)	Chemical Structure	Mol. Wt. (Da)	Melt. Point (°C)	CMC[1] (μM)	Smell[3]
Fatty Acids	Caproic Acid ($C_6H_{12}O_2$)		116.2	-3.4	N.D.[2]	Strong
	Caprylic Acid ($C_8H_{16}O_2$)		144.2	16.5	N.D.	Mod.
	Capric Acid ($C_{10}H_{20}O_2$)		172.3	31.6	3500	Mild
	Lauric Acid ($C_{12}H_{24}O_2$)		200.3	43.8	900	Minor
Monoglycerides	Monocaproin ($C_9H_{18}O_4$)		190.2	19.4	N.D.	Minor
	Monocaprylin ($C_{11}H_{22}O_4$)		218.3	35.6	N.D.	Minor
	Monocaprin ($C_{13}H_{26}O_4$)		246.3	51.4	600	Minor
	Monolaurin ($C_{15}H_{30}O_4$)		274.4	62.5	60	Minor

[1] CMC – Critical Micelle Concentration in μM units. [2]N.D. – Not Determined.
[3] Smell is qualitatively ranked on the order of strong, moderate, mild and minor.

Fatty acids are hydrocarbon chains and one end of the hydrocarbon chain has a carboxylic acid functional group with a pK_a value around pH 5. Most fatty acid molecules are anionic (deprotonated) around neutral pH conditions, while they are mainly nonionic (protonated) in acidic pH environments such as the stomach. Fatty acids with saturated hydrocarbon chains are generally preferable to work with because saturated fatty acids are more chemically stable and less prone to oxidation-related rancidity. Comparatively, the hydroxyl groups of monoglycerides have very high pK_a values (around 14), thereby remaining nonionic across physiologically relevant pH conditions and rendering them highly stable.

Mechanisms of Action

MCFAs and MCMGs have unique mechanisms of disrupting phospholipid membranes and are principally active in the micellar state [Yoon et al, 2015; Kawakami et al, 2017; Yoon et al, 2019]. MCMGs form micelles at lower concentrations than MCFAs (see Table 1), which helps to explain why MCMGs are often more biologically potent than fatty acids and also why longer chain lengths exhibit more potent inhibitory activity than shorter ones within this group. For example, the C_{12} monoglyceride (glycerol monolaurate, abbreviated as GML) has a lower critical micelle concentration (CMC) value (60 μM at pH 7.4) and typically greater potency than both the C_{12} fatty acid (lauric acid; CMC of 900 μM at pH 7.4) and C_{10} monoglyceride (glycerol monocaprate; CMC of 600 μM at pH 7.4) [Yoon et al, 2017; Valle-Gonzalez et al, 2018], see Figure 1 [Jackman et al, under review]. Another important consequence of MCFAs and MCMGs targeting pathogenic membranes is that it is more difficult for susceptible pathogens to develop resistance to these compounds. It is generally acknowledged that there is a very high barrier for pathogens to develop resistance to MCFAs and MCMGs [Desbois and Smith, 2010; Schlivert and Peterson, 2012].

MCFAs and MCMGs are antimicrobial agents that can disrupt the phospholipid membrane surrounding membrane-enclosed pathogens such as bacteria and enveloped viruses. In terms of antibacterial activity, the compounds can inhibit bacterial growth (“bacteriostatic”) through disruption of membrane electron transport and energy metabolism or through displacement of cell-surface membrane enzymes and receptors [Yoon et al, 2015]. They can also induce bacterial cell lysis and death (“bactericidal”) [Yoon et al, 2018]. In general, MCFAs and MCMGs exhibit more potent inhibitory activity against Gram-positive bacteria than Gram-negative bacteria. This can be partially explained by the fact that Gram-positive bacteria have simpler, single lipid bilayer cell membrane structures while Gram-negative ones typically have more complex inner and outer membrane structures.

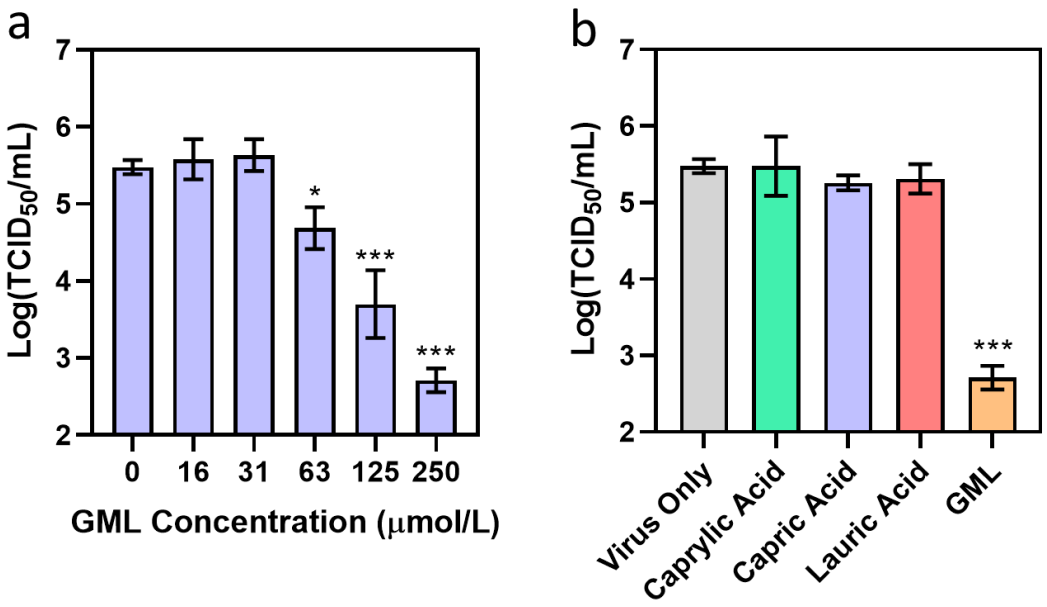


Figure 1. (a) Antiviral activity of GML against African swine fever virus at various concentrations above and below GML's critical micelle concentration of 60 μM. (b) Antiviral activity of selected MCFAs and GML at 250 μM. At 5 mM concentration (20x higher concentration), all MCFAs and GML demonstrated virucidal activity, yielding around 1.2 log reductions in viral infectivity (data not shown). [Jackman et al, 2020 under review]

Pioneering work completed by the Kabara group in the 1970's conducted detailed structure-function studies investigating how fatty acid chain length affects antimicrobial activity [Kabara et al, 172]. In general, capric (C_{10}) and lauric (C_{12}) acids exhibited the highest potencies among fatty acids while the corresponding monoglycerides with equivalent chain lengths were typically even more potent. Nevertheless, it is important to note that different MCFAs and MCMG inhibit different spectrums of pathogens with varying potencies, so appropriate selection is required depending on the pathogen(s) being targeted.

MCFAs and MCMGs can also disrupt a wide range of lipid bilayer-enveloped viruses by damaging and/or effectively destroying enveloped virus particles and compromising infectivity [Jackman et al, 2018]. MCFAs and MCMGs principally exhibit antiviral activity by lysing enveloped virus particles ("virucidal"). They can also disrupt viral protein structures which are required for fusion with host cells, viral replication and re-assembly and protection of the viral RNA or DNA, likely by destabilizing the lipid membranes which support these proteins. On the other hand, MCFAs and MCMGs are inactive against non-enveloped viruses. The list of viruses susceptible to MCFAs or MCMGs includes vesicular stomatitis, herpes simplex, visna, respiratory syncytial, parainfluenza type 2, avian influenza, and ASFv [Thormar et al, 1987; Hilmarsson et al, 2007; Hariastuti, 2011; Sola et al, 1986]. More recent studies demonstrated that they also exhibit strong antiviral activity against other swine-specific viral pathogens, such as

PRRSv and PEDv which contain lipid bilayer envelopes that are necessary for structural integrity and infectivity [Du et al, 2017; Lee, 2015].

Membrane Biophysics

To date, the main scientific approach to study MCFAs and MCMGs has involved empirical testing in microbiology laboratories. The key questions asked by researchers have been whether certain MCFAs or MCMGs inhibit the virus or bacterium under investigation and, if so, how potent is the inhibitory activity? While researchers have long known that the inhibitory activity of MCFAs and MCMGs is associated with viral and bacterial membrane damage, it has proven far more challenging to understand why different MCFAs and MCMGs exhibit varying degrees of inhibitory activity.

One recent solution is an engineering platform called the supported lipid bilayer (SLB), which mimics the basic structural properties of biological membranes. Using SLB technology, researchers are now able to directly study the molecular-level interactions of MCFAs and MCMGs with lipid membranes [Kawakami, 2017]. SLB experimental capabilities revealed that MCFAs and MCMGs interact with lipid membranes in different ways depending on the molecular properties of the tested MCFAs or MCMGs, such as molecular length, shape, and charge. It is possible to conduct concentration-dependent experiments using SLB platforms and to rapidly determine the lowest concentration at which compounds exhibit membrane-disruptive activity. This is related to the CMC value of the compound and has further been shown to correlate with the minimum inhibitory concentration (MIC) value in antibacterial assays. These methods demonstrate the predictive power and efficiency of engineering technologies and will soon allow for the replication of the specific membrane properties from bacteria or viruses and testing of tailor-made mixtures of compounds to most effectively, and at the lowest concentrations, disrupt their functional properties.

Anti-Inflammatory Activity

In addition to antimicrobial properties, certain MCFAs and MCMGs also exhibit immunomodulatory properties. For example, GML is known to affect immune cells, especially T cell lymphocytes, due to membrane interactions linked to cell signaling pathways [Zhang et al, 2018]. Zhang et al. demonstrated that GML treatment can also decrease cytokine production *in vitro* and thus GML exhibits immunosuppressive effects that can be useful for anti-inflammatory applications [Zhang et al 2016]. It was suggested that orally administered GML could be useful for reducing gut inflammation *in vivo*, while it has been demonstrated that vaginal applications of GML can reduce inflammation and infection in a Simian Immunodeficiency Virus (SIV) challenge study [Li et al, 2009]. Additional work that our team conducted with Dr. Barry Bradford's group demonstrated a common anti-inflammatory response among lauric acid and two of its derivatives, GML and lauric acid methyl ester. All forms demonstrated similar reductions in NFκβ expression, indicating lower inflammatory responses to a lipopolysaccharide (LPS) challenge in murine macrophages (Figure 2) ([Mamedova et at, 2019].

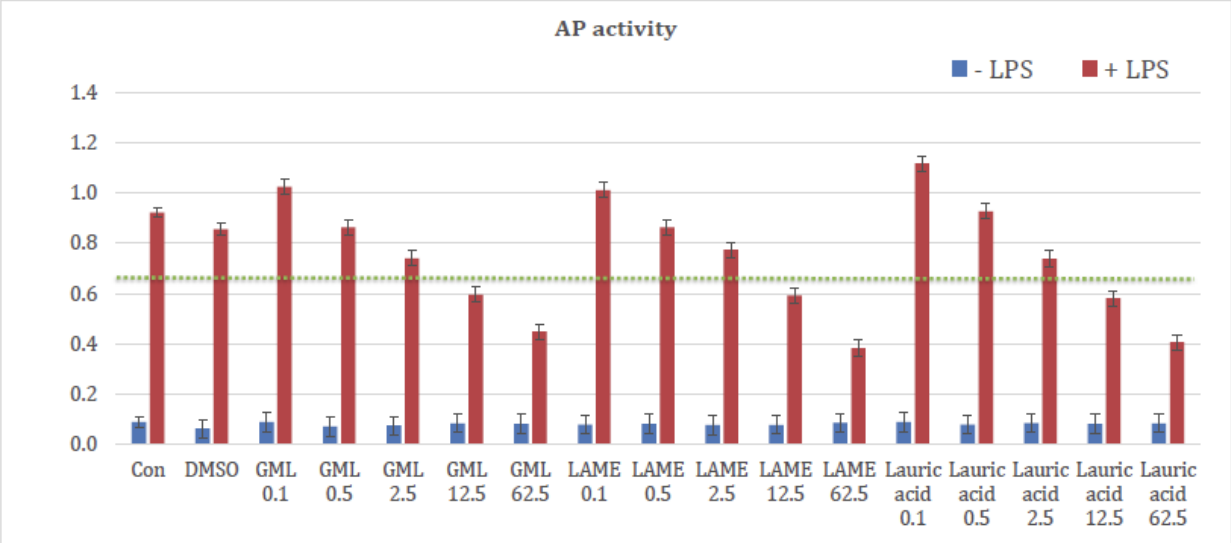


Figure 2. Response of NFκβ to increasing concentrations of lauric acid derivatives following an LPS challenge [Sivinski, et al. 2020].

Growth Promotion

Concomitant with these various beneficial effects to reduce pathogen challenges and inflammation in animals, MCFAs and MCMGs have also been shown to consistently improve animal performance, presumably by preventing the endocrine constraint imposed by high immune stress (Spurlock, 1997) and influencing the balance of gut microbial populations. In a series of studies, Hanczakowska and colleagues investigated the effect of 0.2% caprylic acid and/or capric acid as antibiotic replacements and feed supplements in pigs. They evaluated how diet supplementation with caprylic and/or capric acids affects pig performance, apparent digestibility of nutrients, intestinal microflora, and structure of the ileum [Hanczakowska et al, 2011]. Growth rate was higher (P<0.01) for pigs that received caprylic or capric acids or a combination thereof, along with decreased mortality and increased protein and fiber digestibility when compared to a control group without additive (neither MCFA nor antibiotic). In addition, *Clostridium perfringens* levels in the cecum and ileum were reduced by both fatty acids *(*P<0.01) along with increases in aerobic bacteria *(*P<0.05) and decreases in *Candida* spp. *(*P<0.05) and positive improvements in the structure of the mucosal epithelium in the ileum. Similar effects were observed when fumaric acid (1.5% of the diet) was mixed with either caprylic or capric acid (0.2% of the diet). Fumaric/caprylic acid supplementation led to the largest body weight gains; ADG was 276 g vs. 234 g in the untreated control group (P<0.01) [Hanczakowska et al, 2011]. All three fatty acid treatments led to significant decreases in *Escherichia coli* levels in the digesta collected from the small intestine relative to the untreated control group (P<0.01).

In more recent work, Gebhardt et al. evaluated MCFAs as a dietary additive in nursery pig diets [Gebhardt et al, 2020]. They tested a 1:1:1 blend of caproic, caprylic, and capric acids that were fed at 0, 0.25, 0.5, 1.0, and 1.5% of the diet. Linear dose-dependent improvements in ADG, ADFI, and FCR were noted (P<0.01). Cochrane et al.

also investigated whether MCFAs could be a useful alternative to the antibiotic chlortetracycline in nursery pig diets [Cochrane et al, 2018]. The pigs were challenged with enterotoxigenic *E. coli*, followed by a control diet without additive or one supplemented with (1) 400 g/ton chlortetracycline, or with 1% of the diet composed of an MCFA mixture that contained (2) a 1:1:1 blend of caproic, caprylic, and capric acids, (3) a 12:48:40 blend, or (4) a 4:54:38 blend of the same fatty acids. It was determined that *E. coli*-challenged pigs that received any of the MCFA-containing diets exhibited similar FCR values to those receiving the antibiotic-containing diet.

Livestock Applications

Feed Mitigation

When MCFAs and MCMGs are delivered as feed additives, they can also play an important role in feed pathogen mitigation by inhibiting infectious pathogens (viruses, bacteria), that might be present in the feed and remain viable in the feed matrix for extended periods of time [Dee et al, 2018]. In effect, MCFAs and MCMGs potentially decrease the concentration of infectious pathogens in feed and thereby reduce the probability that animals consuming such feed become infected.

A prominent example of a feed borne pathogen is PEDv. Dee et al. investigated the effectiveness of a 2% MCFA blend comprised of caproic, caprylic and capric acids (1:1:1 ratio) to inhibit PEDv contamination of various classes of swine feed ingredients [Dee et al, 2016]. It was determined that the MCFA blend reduced mean PEDv viral loads in the feed ingredients, as indicated by viral RNA concentrations (viral genome copies) relative to the levels found in the negative control groups treated only with saline solution ($P<0.05$). Subsequent inoculation of piglets with PEDv-contaminated ingredients caused infection, as indicated by detectable PEDv in the small intestine, viral shedding in feces, mild diarrhea, and anatomical changes. By contrast, all piglets inoculated with MCFA-treated, PEDV-contaminated feed ingredients showed no evidence of PEDv infection and the MCFA blend performed equally as well as formaldehyde in the piglet inoculation studies. In an *in vitro* study our team conducted with Dr. Lorin Warnick's group, in which pig feed contained varying levels of GML, 0 to 2% wt/wt, was inoculated with a multi-drug-resistant strain of *Salmonella typhimurium*, and demonstrated a dose-dependent reduction in viable Salmonella after 24 hours of incubation (Figure 3).

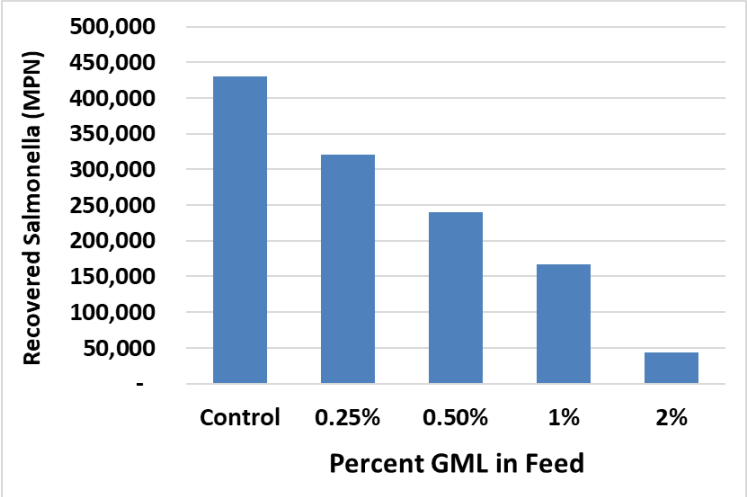


Figure 3. Salmonella (MPN) recovered from feed containing GML and spiked with *Salmonella*. (Warnick and Elrod, unpublished)

Cochrane and co-workers have also systematically studied the mitigating effects of individual MCFAs and combinations thereof on PEDv-contaminated feed samples [Cochrane, 2019]. Test samples included 1% MCFA blend [caproic, caprylic, and capric acids; 1:1:1 ratio] (aerosolized), 1% MCFA blend [caproic, caprylic, and capric acids; 1:1:1 ratio] (non-aerosolized), 0.66% caproic acid, 0.66% caprylic acid, 0.66% capric acid, 0.66% lauric acid, and 1% capric and lauric acid mixture (1:1 ratio). It was determined that the 1% MCFA blends inhibited PEDv to the greatest extent along with caproic, caprylic, and capric acids alone to varying extents ($P<0.05$). This feed pathogen mitigation strategy also protected pigs, who consumed PEDv-contaminated feed, against infection, as indicated by the lack of PEDv in fecal swabs and cecum content.

In related work, which we conducted with collaborators at the Armenian National Academy of Sciences, varying levels of an MCFA mixture and GML (from 0 to 2% wt/wt) were added to pig feed and then spiked with ASFv. At 30 minutes and 24 hours post-inoculation, samples were taken and assayed for viral infectivity, viral DNA and conformationally intact p72 capsid protein. Only GML, at 2%, reduced ($P<0.01$) the infectivity of ASFv at both 30 minutes and 24 hours, though there was a tendency demonstrated at 1% as well. There was no effect of any treatment on the presence of intact viral DNA, which is consistent with the double-membrane envelope structure of ASFv that protects inner genetic material and is markedly more robust than the typical single-membrane structure of other enveloped viruses. Lastly, there was a dose-dependent decline in conformationally intact p72 only in the GML-treated feed samples (Figure 4) [Jackman et al, under review]. This protein is the major capsid protein of ASFv and its conformational change is consistent with GML-induced virus particle disruption, especially since p72 is anchored to viral lipid membranes. As such, disrupting viral lipid envelopes can also impair membrane-associated proteins as well, which underscores the multifunctional impact of GML as a feed additive.

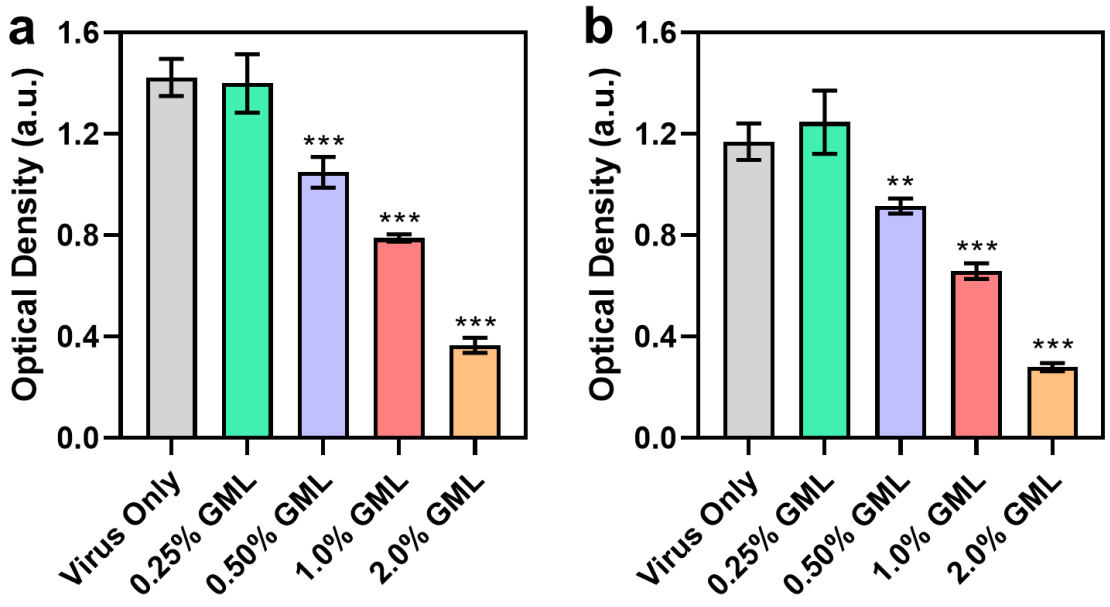


Figure 4. Effect of GML concentration in pig feed on the presence of structurally intact p72 capsid protein, as measured by ELISA, at 30 minutes (a) and 24 hours (b) post-inoculation (Jackman et al, under review).

Altogether, these findings demonstrate that MCFAs and MCMGs can serve as the basis for an effective and natural feed pathogen mitigation strategy. Importantly, their application should be investigated beyond monogastric animals as a potential mitigator of viral and bacterial challenges in pre-ruminants.

Delivery Strategies

Clearly the easiest means to deliver such treatments is via feed. However, when acute disease outbreaks occur, waiting for the next load of feed, or top-dressing treatments onto feed, are not always practical. In these instances, a drinking water or milk-deliverable treatment is the preferred method since it can be rapidly deployed in the initial stages of a disease outbreak. Even when animals go off feed, they typically continue to consume water so this approach would be highly advantageous.

Conclusion

There is tremendous potential for MCFAs and MCMGs as feed additives in livestock production. Continued translation of molecular-level insights into engineered feed additive mixtures might enable the development of precision formulations with varying levels of membrane-damaging activity and pathogen targeting scope. At the same time, further investigation of the virus-killing mechanism of MCFAs and MCMGs in feed and in animals is warranted. Looking forward, this interdisciplinary approach to explore MCFAs and MCMGs as feed additives is highly relevant to a wide range of viral and

bacterial diseases in livestock production and could be extended across many animal species.

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Modeling the Effect of Social Environment on Dry Matter Intake: Time Budget Behaviors and Stocking Density

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Introduction

Nutritional models rely on accurate dry matter intake (DMI), either by measuring or predicting it. Inaccurate DMI predictions can lead to over- and underfeeding of nutrients which translate into lost animal performance and(or) health, inefficiencies in nutrient use, and greater feed costs. Feeding behavior of cattle determines DMI, which is broadly controlled by ruminoreticular fill and chemostatic mechanisms but modulated by the animal's feeding environment (Grant and Albright, 1995). The combination of housing facilities and management routines define the physical and social environment within which cattle consume the feed. Mertens (1994) described these modulatory psychogenic factors and how they influence the animal's behavioral responses to inhibitory or stimulatory factors in the feed or feeding environment separate from the diet's energy or fill value. Social interactions, palatability, and other feed characteristics, as well as learning behavior, are all integral components of psychogenic modulation of DMI (Grant and Albright, 1995). Consequently, actual DMI may be conceptualized as predicted feed intake minus an adjustment for psychogenic factors.

In the future, nutritional models need to incorporate inputs for the feed and feeding environment, such as feeding frequency, stocking density (SD), grouping strategy, and other key psychogenic components to more accurately predict actual DMI. For example, we know that greater stocking density at the feed bunk and free-stall increases aggressive interactions, displacements, and alters meal patterns, rumination, and resting behavior, especially for subordinate cattle (Hill et al., 2009). Currently, research is limited that simultaneously measures feeding and other behavioral responses to the physical and social environment in addition to DMI. Much of the existing data on feeding behavior and DMI were collected using electronic feed bin systems, and it will be a challenge to adapt and apply these data to on-farm systems such as headlocks or post-and-rail feeders.

Previous papers have reviewed the specific influence of cattle grouping and feeding management on feeding behavior and DMI (Grant and Albright, 1995; Grant and Albright, 2001). However, considerable research has occurred since then, particularly for variable stocking density and shorter-term effects on feeding, resting, and rumination behavior. Importantly, these previous reviews did not evaluate the potential importance of resting and feeding behavior and time budgeting as an initial step in DMI prediction and ration formulation. There has been little work on quantifying the management effects on feed intake and creating a mathematical model with these relationships. In order to develop a model that can be used on-farm, we will need to utilize commonly obtained on-farm measures to predict eating time, as it is hard to measure. Therefore, the objective of

this research effort was to create a model that accurately quantifies management decisions on DMI.

Model Development

The model is divided into five sections: 1) behavioral time budget, 2) stocking density measurement, 3) eating time prediction, 4) DMI prediction, and 5) physically effective undigested NDF240 (peuNDF240) adjustment to DMI (Figure 1). Four of the model components focus on management and the social environment, while the fifth component takes advantage of a database generated at Miner Institute of studies where forage source, particle size, and digestibility were varied and fed to high producing Holstein cows (Miller et al., 2020). The model was created using Vensim professional version 7.0a (Ventana Systems Inc., Harvard, MA). This model was designed to be used by dairy farmers or consultants who work with dairy farmers to input specific farm variables to assess the effect of management decisions on DMI, milk production, and behavior.

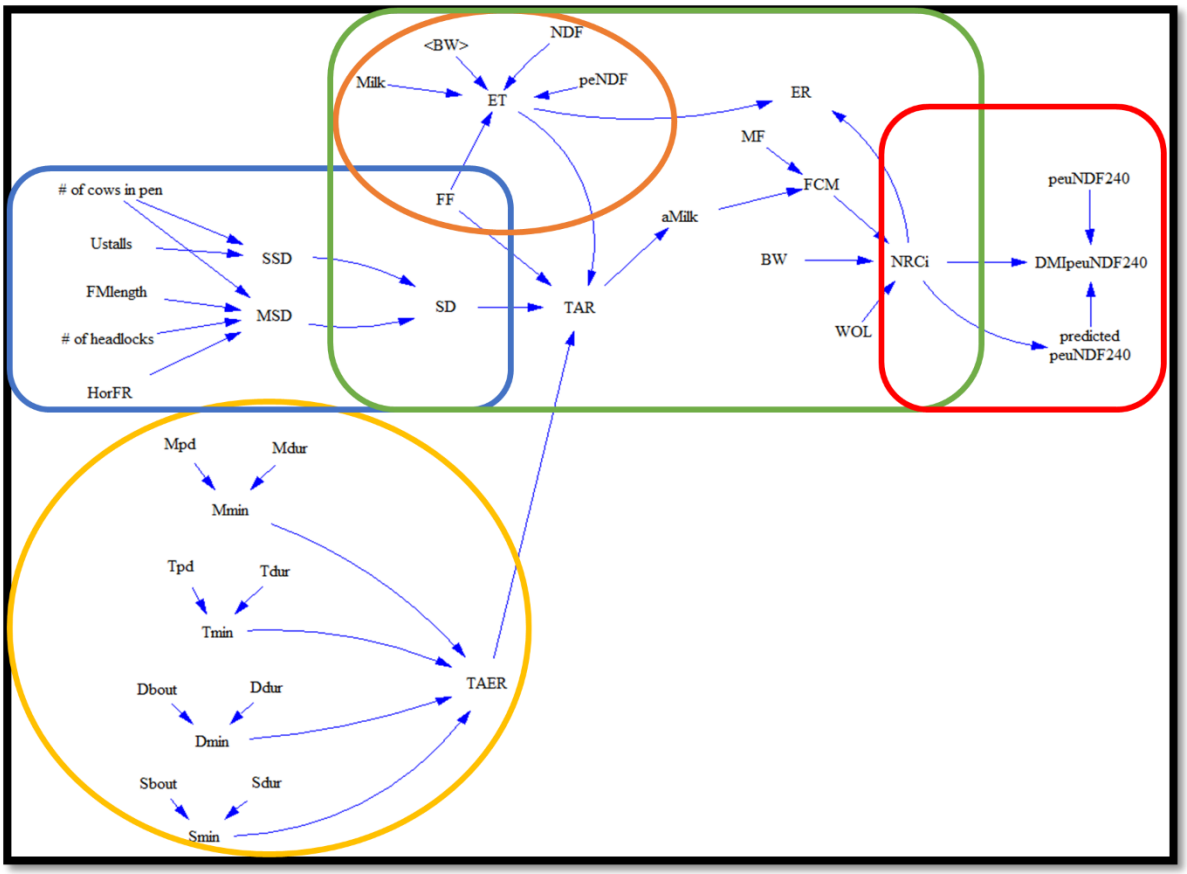

Figure 1. Schematic of the full management model. Yellow circle is the behavioral time budget section; blue rectangle is the stocking density measurement section; orange circle is the eating time prediction section; green rectangle is the DMI prediction section; and red rectangle is the peuNDF240 adjustment to DMI.

The foundation of this model is the behavioral time budget section (Figure 1, yellow circle). This is due to the negative effects that management decisions can have on DMI by not allowing a cow to exhibit her natural behaviors. Grant (2004) reported that a 24-h time budget could be used to describe any deviation from a cow's normal allocation of time to lying, eating, time outside the pen for milking, treatment, drinking, and social interactions. These time durations are intended to be adjusted based on the information for each farm, but it is understandable that not all farms will have this information.

In Figure 1, the section within the blue rectangle allows the calculation of a stocking density using the pen descriptors. The stocking densities are calculated on a free-stall and feed manger basis. The stall stocking density (SSD) is the number of cows in a pen divided by useable stalls (Ustalls) and multiplied by 100. The HorFR variable is used in the manger stocking density variable to choose whether to use the length of feed manger or the number of headlocks based on Friend et al. (1976). The manger stocking density (MSD) for headlocks was calculated as the number of cows in a pen divided by the number of 60-cm headlocks then multiplied by 100. The MSD for feed rails was calculated as the length of the feed rail (FMlength) divided by the number of cows. This was then multiplied by -201.82 and added to 226.37, and this calculation transformed the length of the feed rail per cow into a stocking density based on 100% SD being equal to 0.6 m/cow. Both the SSD and MSD are connected to the SD variable, which selects the largest of the two for the stocking density used for prediction. We decided to use the largest stocking density measurement to represent pens that either have more stalls or manger space; therefore, this approach will account for the resource (stall or manger) with the most competition.

In Figure 1, the eating time prediction is presented within the orange circle. Lying and eating time are the two largest portions of a cow's daily time budget, but they typically cannot be measured easily on-farm. Therefore, it is essential to predict one, so that the other can be calculated by subtraction. In order to allow stocking density to affect lying time, we decided to predict eating time and calculate the lying time. The feeding frequency (FF) variable also influences eating time (ET). If FF was once per day, then there would be no adjustment to ET. If FF was two times per day, then ET would be increased by 3.5%. Finally, if FF was greater than two, then ET would be increased by 10% based on published relationships between frequency of feed delivery and eating time (Philips and Rind, 2001; DeVries et al., 2005; Mantysaari et al., 2006).

In Figure 1, within the green rectangle, the DMI prediction is presented. The time available for rest (TAR) variable was calculated as TAER minus the ET variable. Feeding frequency and SD were used to make an adjustment on TAR. The TAR variable then influenced the adjusted milk (aMilk) variable. We decided to evaluate the relationship between lying time and stocking density and milk yield and built a database using nine studies with 39 treatments (Hill, 2006; Fregonesi et al., 2007; Proudfoot et al., 2009; Krawczel et al., 2012a,b; Winckler et al., 2015; Campbell, 2017). The average SD was 126%, with a minimum of 75% and a maximum of 200%. The DMI and milk yield averaged 22.2 and 40.3 kg/d, respectively. So, in this proposed management model, stocking density affects lying time, which results in an adjustment to the milk yield, which is used

to predict DMI. Finally, the NRCi variable is divided by ET and expressed as kg of DM per minute in the eating rate (ER) variable.

In Figure 1, the peuNDF240 adjustment of DMI is presented within the red rectangle. This adjustment was used to account for situations when the dietary peuNDF240 content negatively affects DMI. The NRCi variable was used to predict a peuNDF240 content in the predicted peuNDF240 variable. Then, if this variable was greater than the peuNDF240 variable, the peuNDF240 intake prediction was used in the DMIpeuNDF240 variable.

Data from studies included in the database for eating time, lying time, adjusted milk, and peuNDF240 intake predictions were analyzed using the MEANS procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC), and were reported as descriptive statistics (mean ± standard deviation; minimum and maximum values). Predictions were created using multiple linear regression (MLR) using the REG procedure of SAS. Mean absolute error (MAE) was calculated as the absolute value of actual observation minus predicted value and was used to assess predictive ability.

Results and Discussion

All variable names used in the equations for the management model are described in Table 1, and the equations in the final model are listed in Table 2. To our knowledge, there has been no previous research that has built a mathematical model to capture the effects of stocking density and feeding frequency on behavior and performance of lactating dairy cattle using behavioral time budgeting as the foundation of the model.

In deciding whether to predict lying or eating time, it was essential to understand which behavior had more importance to the cow. Munksgaard et al. (2005) reported that, when cows are limited in access to feed and rest, lying time was prioritized over eating. The cows compensated for the decreased eating time by increased eating rate to maintain DMI (Munksgaard et al., 2005). Since cows can compensate for less time available for eating by increasing their eating rate to maintain DMI, we decided to predict it. In contrast, unfortunately, the cow that is rest-deprived cannot compensate and will be negatively affected.

To assure that the eating time prediction would be applicable on a farm we decided to use measures that are routinely quantified on-farm. The on-farm measures we selected were dietary NDF content, peNDF content, milk yield, and BW. Dado and Allen (1994) and Roseler et al. (1997) reported a positive relationship between milk yield and DMI. This makes sense as the amount of energy intake is one of the main factors that affect milk production. Oba and Allen (2000a,b) reported that cows fed low NDF concentration diets had greater DMI and spent less time eating compared to cows fed high NDF concentration diets. Dado and Allen (1994) also reported a moderate positive correlation between eating time and DMI. Based on previous research, we were confident of variable selection, but needed to create and validate the DMI prediction equation.

Table 1. Description and units of the variables used in equations in the management model.

Variable	Unit	Description
# of cows in pen	n	Number of cows in pen
# of headlocks	n	Number of headlocks for a pen
aMilk	kg	Predicted milk using stocking density with an adjustment for feeding frequency
BW	kg	Average body weight of cows in pen
DMIpeuNDF240	kg	Predicted dry matter intake using physically effective undigested neutral detergent fiber at 240-h
Dbout	n	Number of drinking bouts per day
Ddur	min	Average length of the drinking bouts
Dmin	min	Product of drinking bouts and drinking duration
ER	kg/min	Eating rate based on NRC (2001) DM intake and eating time
ET	min/d	Predicted eating time
FCM	kg/d	4% fat-corrected milk
FF	n	Number of feedings per day
HorFR	1 or 0	Whether the pen has headlocks or feed rail
FMlength	m	Length of feed rail for a pen
MSD	%	Stocking density of manger
Milk	kg/d	Average milk production of a pen
MF	%	Average milk fat content of a pen
Mdur	min	Average length of milking
Mmin	min/d	Product of milking duration and milkings per day
Mpd	n	Number of milkings per day
NDF	% of DM	Neutral detergent fiber content of the diet
NRCi	kg/d	NRC intake prediction
peNDF	% of DM	Physically effective NDF content of the diet
peuNDF240	% of DM	Physically effective undigested NDF at 240 h content of diet
Predicted peuNDF240	% of DM	Predicted physically effective undigested NDF at 240 h content of the diet
Smin	min/d	Product of social bouts and social duration
Sbout	n	Number of social bouts per day
Sdur	min	Average length of social bouts
SSD	%	Stall stocking density of a pen
SD	%	Larger of the stall stocking and manger stocking density
TAER	min/d	Time for eating and resting
TAR	min/d	Resting time with feeding frequency and stocking density adjustment
Tdur	min/d	Product of treatment minutes and treatments per day
Tmin	min	Average length of treatments
Tpd	n	Number of treatments per day
Ustalls	n	Stalls cows can use in a pen
WOL	n	Average week of lactation of the pen

We used six studies of high producing dairy cows fed high and low forage diets containing different sources of forages and varying forage particle sizes to create prediction equations for eating time (Kononoff et al., 2003; Cotanch et al., 2014; Miller et al., 2017; Smith et al., 2018; Coons et al., 2019; Miller et al., 2020). The MLR analysis to predict eating time accounted for 68% of the variance using NDF content, peNDF, BW, and milk yield. A large proportion of the accounted variance for eating time was from the milk yield (37.5%) and NDF content (25.3%). Our results agree with previous research that also found milk and dietary fiber content to be important when predicting DMI (Dado and Allen, 1994; Roseler et al., 1997; Oba and Allen, 2000a,b).

To test the predictive ability of the equation from MLR, we compiled 13 published studies with 50 treatments using lactating Holstein dairy cows that included DMI, milk yield, eating time, BW, NDF content, and peNDF content (Grant et al., 1990; Beauchemin et al., 2003; Yansari, et al., 2004; Yang et al., 2006, Yang et al., 2007; Yang et al., 2009; Hart et al., 2013; Hart et al., 2014; Farmer et al., 2014; Campbell et al., 2015; Crossley et al., 2017; Campbell et al., 2017; Crossley et al., 2018). The mean absolute error (MAE) of eating time was calculated using the prediction equation from MLR using the 13 published studies split into different groups (all, multiparous, primiparous, and mixed). The eating time prediction equation had the best predictive ability for multiparous cows with a MAE of 30 min/d. In contrast, the eating time prediction equation for the other groups had a similar MAE of 41 min/d. To our knowledge, there has not been previous research that attempted to predict eating time using on-farm measures. Our eating time prediction had a good initial predictive ability, with an average MAE of 41 min/d, however, there is a need to continue to improve this prediction.

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Table 2. Equations used in the management model[1].

Variable	Unit	Description
aMilk	kg	0.04065 x TAR + 11.2444
DMIpeuNDF240	kg	IF THEN ELSE(peuNDF240 > predicted peuNDF240, (-0.9798 x peuNDF240)+32.848, NRCi)
ER	kg/min	NRCi / ET
ET	min/d	IF THEN ELSE(FF = 1, (-70.3442 + (BW x -0.3241) + (Milk x 4.04145) + (NDF x 13.2501) + (peNDF x -3.06001)), IF THEN ELSE(FF = 2, 1.035 x (70.3442 + (BW x -0.3241) + (Milk x 4.04145) + (NDF x 13.2501) + (peNDF x -3.06001)), IF THEN ELSE(FF > 3, 1.1 x (-70.3442 + (BW x -0.3241) + (Milk x 4.04145) + (NDF x 13.2501) + (peNDF x -3.06001)), 1)))
FCM	kg/d	(0.4 x aMilk) + (15 x (aMilk x (MF / 100)))
MSD	%	IF THEN ELSE(HorFR = 1, # of cows in pen / # of headlocks x 100, (FMlength / # of cows in pen) x -204.818 + 226.373)
NRCi	kg/d	((0.372 x FCM) + (0.0968 x BW^0.75)) x (1-EXP(-0.192 x (WOL+3.67)))
Predicted peuNDF240	% of DM	-((NRCi - 32.848) / (0.9798))
SSD	%	# of cows in pen / Ustalls x 100
SD	%	MAX(MSD, SSD)
TAER	min/d	1440 - (Dmin + Mmin + Smin + Tmin)
TAR	min/d	IF THEN ELSE(FF > 4, ((-0.00191 x SD + 1.19199) x (TAER - ET)) x 0.88, ((-0.00191 x SD + 1.19199) x (TAER - ET)))

[1]All other variables are as defined in Table 1.

The eating time prediction equation was used in the ET variable. The variable TAR was calculated by subtracting the ET variable from the TAER variable. We then made an adjustment for stocking density and feeding frequency. Stocking density was defined as the number of animals per resource, such as stall or headlock, usually expressed as percent for stalls and meters per cow for manager space. Overstocking is defined as having more animals than resources and has become a common practice on dairy farms (von Keyserlingk et al., 2012). The only variable in the model directly affected by stocking density is TAR, and this decision was based on previous research that described the relationship between stocking density and daily resting time.

Several short-term studies have investigated the effect of overstocking on DMI, and in general there is no effect (Batchelder, 2000; Collings et al., 2011; Krawczel et al., 2012b; Campbell et al., 2015; Wang et al., 2016; Campbell et al., 2017; Crossley et al., 2017). Cows that are overstocked above 130% spent less time lying compared to cows stocked at 100%, and importantly, overstocking did not affect eating time (Fregonesi et al., 2007; Hill et al., 2009; Krawczel et al., 2012b; Campbell et al., 2015; Campbell et al., 2017). The extra time created by reduced resting with overcrowding was not spent eating,

but rather standing idle in the alley (Fregonesi et al., 2007; Hill et al., 2009; Krawczel et al., 2012b; Campbell et al., 2015; Campbell et al., 2017). This increased standing time can have negative effects on health such as poor hoof health, greater serum cortisol, and lower growth hormone, which could lead to lower milk production (Munksgaard and Lovendahl, 1993; Singh et al., 1993; Grant, 2004; Cooper et al., 2007). Although overstocking did not affect milk yield, this could be due to the studies being short-term in nature (Krawczel et al., 2012b; Campbell et al., 2015; Campbell et al., 2017; Crossley et al., 2017). There is a need for future overstocking research to focus on the longer-term effects on DMI and milk yield.

Since the previous research did not show a direct relationship between overstocking and DMI, we decided to use the relationship between lying time and stocking density. In Figure 1, we present the relationship between SD and relative response for lying time. The MLR analysis to predict lying time accounted for 76% of the variance using stocking density (Figure 1). Our results agree with Grant (2015), and we were able to account for more variation.

The TAR variable was adjusted by FF as any FF greater than or equal to five times per day may reduce lying time by 12% (Philips and Rind, 2001; DeVries et al., 2005; Mantysaari et al., 2006). There is relatively little published research on feeding frequency and even less on its effect on cow behavior. The TAR variable was adjusted by SD and FF variables and was then used to predict a milk yield in the aMilk variable. Again, we used the database to re-evaluate this relationship. The MLR analysis to predict milk yield accounted for 36% of the variance using lying time (Figure 2). Our results were in agreement with Grant (2015), and we were able to account for more variation. So, we used this revised equation based on our database in the aMilk variable. Unfortunately, there is a limited amount of published data for the effect of overstocking on lying time and lying time on milk yield, which limits our ability to check the predictive ability of our predictions.

The aMilk variable was used to calculate a FCM value which is used in the NRC (2001) DMI prediction in the NRCi variable. The decision to use the Dairy NRC (2001) equation was based on its common use in the dairy industry. The ER variable was calculated using the ET and NRCi variables. As stated earlier, cows that are overstocked will increase their eating rate to maintain DMI, so the ER variable could be used to assess how well the model captures this behavior. There are limited data on eating rate due to the difficulty and cost of measurement. Future research needs to focus on effects of stocking density on chewing behaviors such as eating and ruminating.

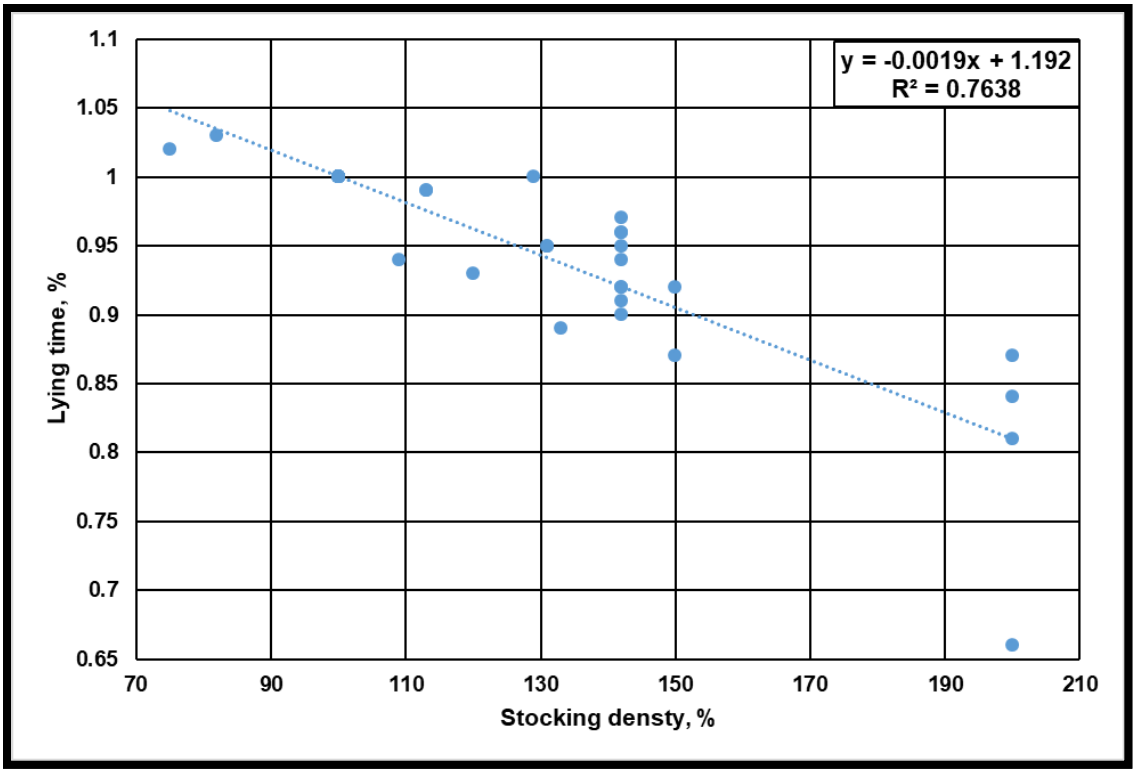

Figure 1. Relationship between stocking density and lying time for management model.

Recent research has focused on fiber characteristics such as particle size and indigestibility and their effects on DMI (Smith et al., 2018). Smith et al. (2018) investigated the relationship between peNDF and uNDF240 in lactating dairy cow diets. They created a measure called peuNDF240, which is the product of the dietary pef and uNDF240 and was intended to integrate the effects of particle size and NDF indigestibility into one number. The peuNDF240 was highly related to DMI and chewing behavior (Smith et al., 2018). To explore this new measure's relationship with DMI further, we created a database with five studies with 16 treatments (Cotanch et al., 2014; Miller et al., 2017; Smith et al., 2018; Coons et al., 2019; Miller et al., 2020).

The MLR analysis to predict DMI accounted for 60% of the variance using peuNDF240 (Figure 3). This result agrees with the findings of Smith et al. (2018) and can be used to adjust DMI dependent on the peuNDF240 content of the diet. In the management model, we used the NRCi variable to predict a peuNDF240 content. If the dietary peuNDF240 content was greater than the predicted peuNDF240, then we used the regression equation created from the database. There is limited research using these new fiber measures, and it is important to restrict these inferences to similar diets (corn silage with hay and fibrous byproducts).

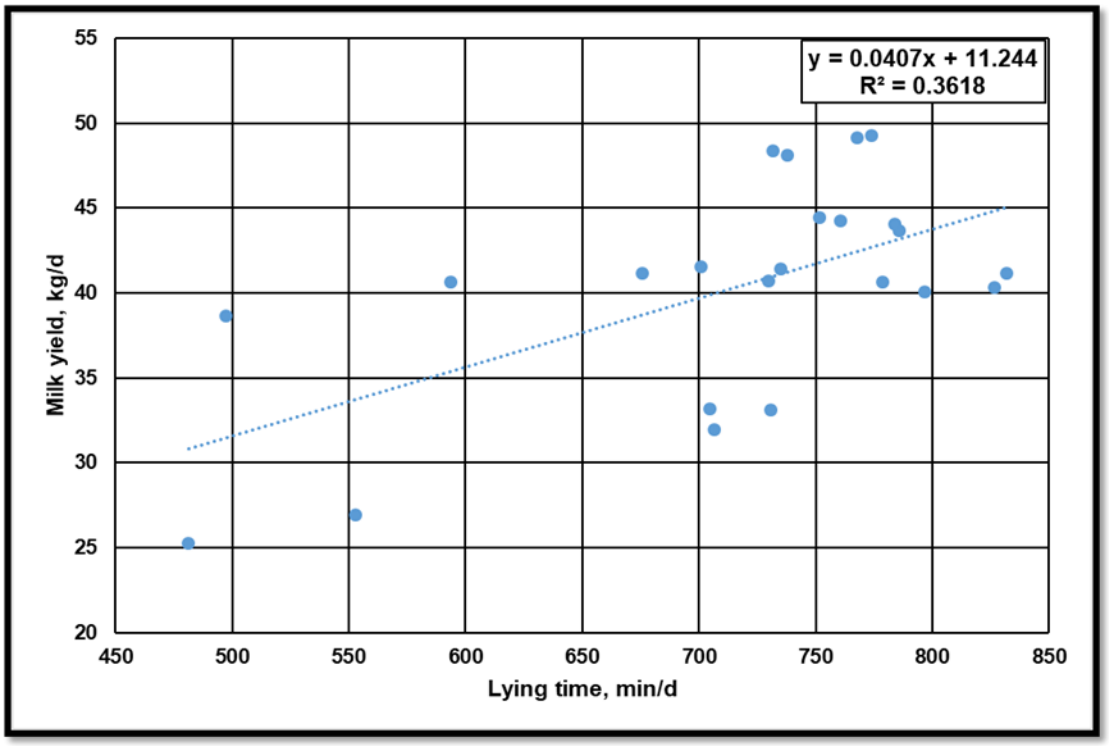

Figure 2. Relationship between lying time and milk yield for management model.

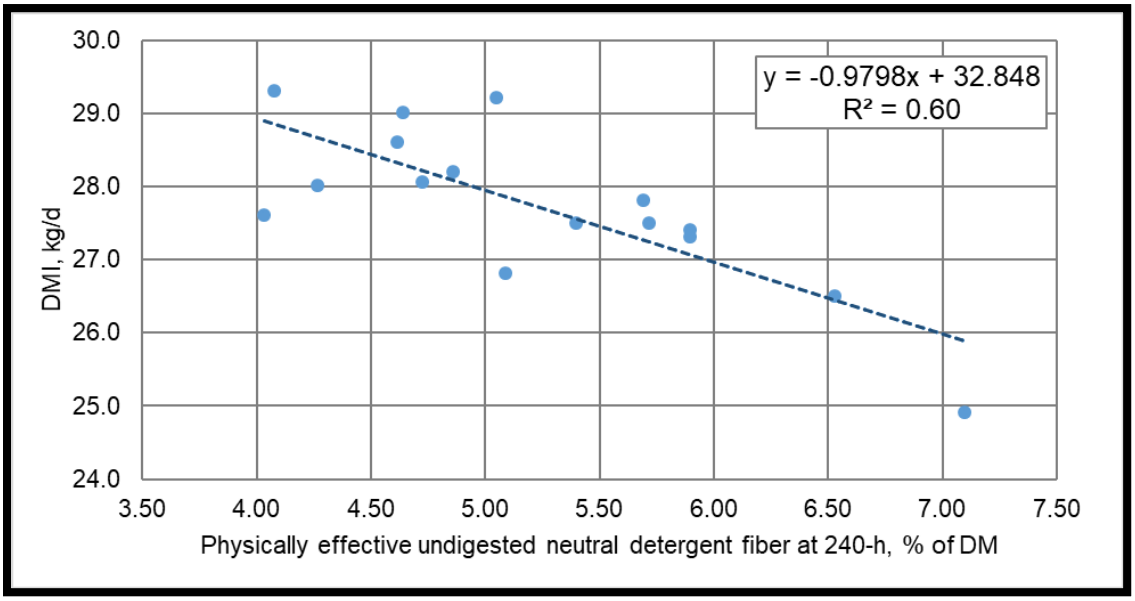

Figure 3. Relationship between physically effective undigested neutral detergent fiber at 240-h and dry matter intake for management model.

Conclusions

This study's objective was to create a model that accurately quantifies the effects of stocking density and feeding frequency on behavior and performance of lactating dairy cattle. The foundation of the management model is the time budget with lying and eating being the most significant time allotments. The eating time was predicted using common on-farm measures (NDF content, peNDF, BW, and milk yield) and had a good predictive ability with a mean absolute error of 41 min/d. Stocking density affected lying time, which accounted for 76% of the variance in lying time. The adjusted lying time was then used to predict a milk yield, which accounted for 36% of the variance in milk yield. Intake was affected by peuNDF240 content of the diet as the peuNDF240 increased DMI decreased. The peuNDF240 accounted for 60% of the variance in DMI. The management model appears to have potential to be a useful tool for producers and consultants, although more data and research are needed to validate the model. We expect to conduct this validation over the next year.

Take Home Messages

- Eating time can be predicted using on-farm measures.
- Lying time has a large influence on milk production.
- The peuNDF240 content of the diet increases the dry matter intake decreases.

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Advances in the Determination of Nutrient Requirements of the Pre-weaned Dairy Calf

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Introduction

There is growing evidence concerning the importance of optimizing growth and body composition of dairy replacements to foster future productivity (Soberon and Van Amburgh, 2013). As a consequence, there is a need and opportunity to improve and further develop available nutritional models, like the Cornell Net Carbohydrate and Protein System (CNCPS, Van Amburgh et al., 2015), to improve the precision of diet formulation and optimize nutrient utilization in this important group of animals.

Although not technically classified as a nutrient, energy is the variable first considered by nutritionists when determining the adequacy of a diet to support a given level of animal production (Blaxter, 1962). For this reason, understanding nutritional energetics is imperative in the development of a system that is designed to predict energy balance of immature dairy heifers.

Likewise, during the first stages of development, the calf's amino acid (**AA**) needs are high as indicated by the rapid and lean growth unique to this period of life (Van Amburgh et al., 2019). Furthermore, protein accretion has been proposed as the primary factor enhancing future production of the pre-ruminant heifer (Soberon and Van Amburgh, 2013). Therefore, provision of the nutrients necessary to support proper growth and composition during this phase is critical for the dairy heifer, and the dairy industry. Current guidelines from NRC (2001), describe the calf's requirements in terms of apparently digestible protein, a less refined measure than the grams of Lys or Met per day recommended for lactating cows by the same system. In addition, this approach assumes a constant composition of gain and a static efficiency of protein utilization over the growing period.

In their analysis, Van Amburgh and Drackley (2005) suggested that a more dynamic approach could improve the predictive capability of current models and allow for greater refinements based on source of energy and AA. With this in mind, the objective of the present work was to generate a system of equations to quantify energy, N and AA requirements, supply and utilization, which, when integrated into a formulation model could be used to assess energy, N and AA balance in young pre-weaned dairy calves. This was accomplished utilizing body composition and nutrient utilization data generated over the last 20 years and employing updated AA analysis methods to ensure appropriate recovery of AA.

Materials and Methods

For the development of equations, six comparative slaughter studies were used (Bascom et al., 2007; Diaz et al., 2001; Tikofsky et al., 2001; Blome et al., 2003; Bartlett et al., 2006; Mills et al., 2010), while four different body composition studies (Diaz et al, 2001; Blome et al., 2003; Bartlett et al., 2006; Bascom et al., 2007) were used to develop equations for N and AA requirements since they provided different levels of N intake and N digestibility. The complete data set included nutrient intake, full BW, empty BW (**EBW**), EBW gain (**EBWG**) and body composition (DM, N, fat and ash) of 239 animals (24 Jerseys and 215 Holsteins) allocated in 35 nutritional treatments of different feeding levels, dietary nutrient composition or both. Three of these treatments consisted of feeding whole milk, while the rest fed milk replacers based on whey proteins.

The energy content of milk replacers was measured with a bomb calorimeter in all studies, while for whole milk, energy was estimated empirically using the chemical composition. Metabolizable energy intake (**MEI**) was calculated as the intake energy multiplied by the metabolizability coefficients. Diaz et al. (2001) and Blome et al (2003) reported measured energy metabolizability values and these were applied to their respective treatments. For the other treatments and calculations, 0.91 was used as the value for conversion to MEI of the intake energy (Van Amburgh and Drackley, 2005). The gross energy from whole milk was estimated assigning the energy contents (Mcal/kg) of 9.21, 5.86 and 3.95, to fat, protein and lactose, respectively (NRC, 2001). The carbohydrate content, presumably as lactose, was calculated by difference, subtracting fat, CP and ash fractions from the total DM. Energy in tissues was determined by bomb calorimetry in all studies except for that of Bartlett et al. (2006) in which tissue energy was computed assigning energetic values to fat (9,250 kcal/kg) and crude protein (5,104 kcal/kg) in the body.

The EBW was calculated as the sum of the body fractions, which for most studies consisted of carcass, blood and organs, and head, hide, feet and tail, with the exception of Blome et al. (2003) who partitioned the empty body into viscera and viscera-free carcass. Chemical components were assigned to the respective fraction and added to represent the final empty body composition. Retained energy (**RE**) and N (**RN**) in the body were calculated by difference between their content in the final EBW and that in the initial EBW, as indicated by the comparative slaughter method (Lofgreen, 1964). Initial EBW was estimated by extrapolating the BW composition of a reference group harvested at the beginning of each study to the initial BW of the animals assigned to the different treatments.

Analyses were performed using R (v. 3.6.0; R Core Team, 2019). Simple, multiple linear, and nonlinear regressions were fitted using the ‘nlme’ function from the ‘nlme’ package (Pinheiro et al., 2020). Regressions were performed as mixed-effect models, where fixed effects varied by equation, while the random effect of study and that of treatment nested within study or that of study alone were included, when individual calf or treatment average data were used, respectively. When using treatment averages, data were weighted in the variance structure using the square root of the number of individuals per treatment.

Preplanned comparisons were performed for parameters related to energy metabolism between calf breeds. Parameter comparisons were made using the 'pairs' function from the 'emmeans' package (Lenth et al., 2019). Significance was declared at $P \leq 0.05$. Predictive models were assessed using the root mean squared error (**RMSE**) as percentage of the observed mean, mean and slope bias as a percentage of the mean squared error (MSE; Bibby and Toutenburg, 1978), and concordance correlation coefficients (**CCC**; Lin, 1989).

Energy Requirements

Maintenance

Adopting 0.75 as the allometric exponent to determine metabolic EBW (**MEBW**, $kg^{0.75}$), net energy requirement for maintenance ($\mathbf{NE_m}$) was estimated by extrapolation from the relationship between heat production (**HP** = MEI - RE) and MEI. Using this relationship, the point at which MEI and HP were equal was considered the ME requirement for maintenance ($\mathbf{ME_m}$). The partial efficiency of use of ME for maintenance ($\mathbf{k_m}$) was calculated as the ratio between NE_m and ME_m.

The mean ME_m for Jerseys (137.66 kcal/kg of MEBW) was 35% higher than for Holstein calves (102.17 kcal/kg of MEBW), which agrees with the differences found in ME_m between dry, non-pregnant mature cows of these breeds (Solis et al., 1988). The higher ME_m of Jerseys appears to be related to both their higher NE_m (85.5 ± 9.85 vs 73.1 ± 2.20 kcal/kg of MEBW) and lower k_m (0.62 vs 0.72). Brody (1945) also reported a greater resting heat production (NE_m) per unit of surface area in Jerseys compared to Holsteins, suggesting a difference in the metabolic rate between these breeds. The lower k_m of Jerseys might be due to the greater heat loss because of a larger surface area relative to BW and differences in body proportions compared to Holsteins. Using Brody's (1945) equation to estimate surface area ($m^2 = 0.15 \times kg^{0.56}$ of BW), Jersey calves had 37% more surface area per kg of BW than Holsteins in the current data set. Thus, although not different in this analysis, the numerical differences in ME_m between breeds might have biological and practical significance for the feeding and management of calves of these breeds, as undersupplying energy could affect the health and growth of the young Jersey. Further, a teleological argument can be made that the composition of milk from Jersey cows suggests higher energy intake from fat is required by the calf to account for greater heat loss.

In addition, because energy is expressed in MEBW basis as opposed of the commonly used metabolic full or live BW, which includes the gut fill mass, the current estimates needed to be scaled for proper comparison with the literature. In the present data, the ratio of EBW to full BW was 0.93 ± 0.002. The NE_m and k_m estimated here for Holstein calves (69 $kcal/kg^{0.75}$ of BW and 0.72) are lower than the values adopted by the Dairy NRC for preruminant calves (87 $kcal/kg^{0.75}$ of BW and 0.85, respectively). However, the resulting ME_m were in close agreement (97 and 100 $kcal/kg^{0.75}$ of BW, respectively) and well within the range of 90 to 110 $kcal/kg^{0.75}$ of BW described by Davis and Drackley (1998).

Data on the energy metabolism of Jersey calves are scarce. However, one study reported that both NE_m (104 kcal/kg$^{0.75}$ BW) and MEm (95 kcal/kg$^{0.75}$ BW) were similar between Jersey and Holstein calves (Holmes and Davey, 1976). Although these estimates are close to the ME_m coefficient calculated in this study for Holsteins, the values for NE_m, k_m, and the estimates for Jersey calves are not similar.

The estimated ME_m coefficients inevitably contain some energy cost related to physical activity and thermoregulation given that animals were managed under variable environmental conditions similar to normal production settings. Thus, coefficients estimated herein could be used to calculate ME_m at thermoneutrality and adjusted by adding the energy requirement for thermoregulation when animals are outside of their thermoneutral zone. The additional energy expenditure for thermoregulation could be made with currently available methods and estimates (Schrama et al., 1993)

Efficiency of ME utilization for growth

The ME available for growth (**ME_g**) was calculated by difference between MEI and ME_m, while the fixed partial efficiency of ME utilization for growth (**k_g**) was considered to correspond to the slope of the linear regression between RE and ME_g with no intercept. Estimates of fixed k_g indicate that Holstein calves (0.55 ± 0.013) are more efficient utilizing ME above maintenance for growth than their Jersey counterparts (0.39 ± 0.058; $P < 0.01$). The difference in k_g between breeds in the preruminant state is puzzling since the dissimilarities identified in their ME_m requirements were already accounted for in the ME_g estimation. Moreover, the digestion and metabolizability of milk and milk replacers does not appear to be affected by the breed of the calf (Blaxter and Wood, 1952; Diaz et al., 2001; Bascom et al., 2007). An additional analysis suggested the difference in k_g between breeds is due to a lower energetic efficiency of fat deposition in Jersey calves (Molano, 2020). The k_g determined for milk-fed Holsteins is close to the 0.60 previously derived by Van Amburgh and Drackley (2005), reinforcing their conclusion that the coefficient of 0.69 currently used by the NRC calf sub-model (2001) is not appropriate.

The efficiency of ME utilization has been challenging to describe and predict in growing animals, as it is affected by many factors (Nozière et al., 2018) and cannot be considered as fixed, as showed in Figure 1A where the treatment average k_g were plotted against the treatments identification number (1 to 35). This has been observed by others and in the growing ruminant k_g has been dynamically estimated based on the quality of the diet (Alderman and Cottrill, 1993) or, more recently, as a function of composition of gain (Williams and Jenkins, 2003; Nozière et al., 2018). Given the multifactorial nature of the variability around k_g and the limitation of the current approaches to describe it, alternative methods were explored. From these evaluations, the approach that accounted for the most variation was relating k_g to the kcal of ME available for growth on MEBW basis, as shown in Figure 1B. Two distinct patterns were identified corresponding to each breed and they were best described by a loglogistic function with two parameters. This indicates that the efficiency at which animals use energy for growth declines as more energy is available once maintenance requirements are met, in agreement with the law of diminishing returns.

This observation challenges the classical approach which estimates k_g by linear regression assuming that both MEm and k_g are constant regardless the level of nutrient intake. In fact, because the supply of ME_g relative to MEBW could be considered a measure of feeding level, the apparent "decrease" in efficiency of use for growth could be masking the increasing energy maintenance costs associated with a higher level of feeding. The variation in the apparent requirements of maintenance has been recognized since the early stages of nutritional energetics (Baldwin and Bywater, 1984) and different approaches have been proposed to account for the effect of feeding level in a variable representation of the requirements for maintenance in cattle (Ferrell and Oltjen, 2008) and veal calves (Labussière et al., 2011). However, the present approach followed the conventional scheme of energy fractionation proposed by the factorial method, considering the increase in apparent maintenance with level of nutrient intake as a cost of production and allocating the additional energy to a variable estimate of the efficiency of ME use for growth ($_{var}k_g$), while setting requirements of maintenance constant.

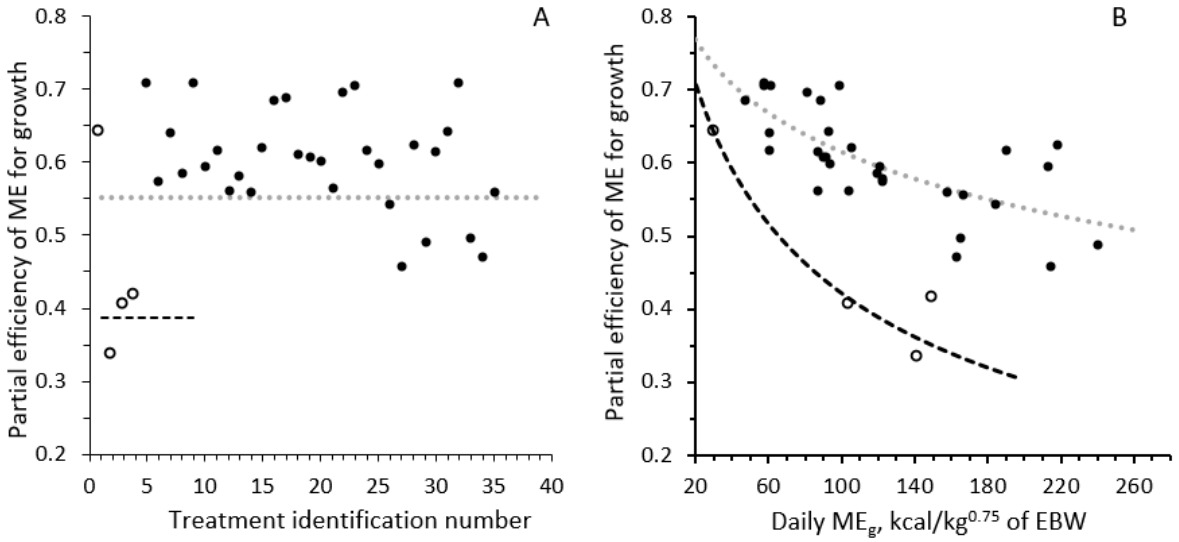

Figure 1. The partial efficiency of ME utilization for growth (k_g) as a function of treatment identification number (A) and feeding level (B) for milk-fed Jersey (open circle, dashed line) and Holstein calves (closed circle, dotted line). In panel A lines indicate the estimated fixed k_g for Jersey (0.39) and Holstein calves (0.55). In panel B lines indicate the loglogistic function describing the relationship between k_g and feeding level expressed as daily ME available for gain (ME_g) on MEBW basis. Dotted line [$_{var}k_g = 1/(1 + \exp(0.46 \times (\log(ME_g) - 5.64))$]; dashed line [$_{var}k_g = 1/(1 + \exp(0.74 \times (\log(ME_g) - 4.18))$]. Circles represent treatment means.

Prediction of Retained Energy

The effect of using a fixed or variable approach to determine k_g on the ability to predict energy balance was evaluated by predicting RE from ME_g [i.e. $RE = ME_g \times k_g$ (as fixed or variable)]. Measures of model adequacy are in Table 1. There were significant slope and mean biases identified when the fixed k_g was employed, under-predicting RE at low energy retentions and over-predicting it at higher levels of energy retention.

Alternatively, the variable k_g computed as a function of feeding level ($_{var}k_g$) improved the precision and accuracy of the RE prediction for which no mean or slope bias were observed. Therefore, the later approach was adopted. Since the variation in k_g is being accounted for with energy intake, it makes sense to use the resulting $_{var}k_g$ to convert ME_g to predicted RE.

Table 1. Model adequacy statistics for the prediction of retained energy (kcal/d) using ME above maintenance (ME_g) and a fixed or variable efficiency of ME utilization for gain (k_g) and by different predictive equations.

Item[1]	$ME_g \times k_g$		Equation		
	Fixed k_g	Variable[2] k_g	Toullec[3]	Van Amburgh and Drackley[4]	Proposed[5]
Observed mean	1,386.87	1,386.87	1,386.87	1,386.87	1,386.87
Predicted mean	1,328.67	1,398.76	1,537.95	1,242.45	1,389.03
RMSE, % mean	19.38	17.60	26.12	21.05	16.53
Mean bias, % MSE	4.69	0.24	17.40	24.48	0.01
Slope bias, % MSE	8.58	0.22	32.74	9.63	0.22
CCC	0.93	0.94	0.89	0.92	0.94

[1]Model evaluation criteria included root mean squared error as a percent of observed mean (RMSPE), mean and slope bias as a percent of mean squared prediction error (MSPE), and concordance correlation coefficient (CCC).
[2]Variable efficiencie of ME utilization as function of feeding level
[3]Toulec (1989), RE = (0.84 × $LW^{0.355}$ × $LWG^{1.2}$) × 1000. Liveweight (LW) and daily LW gain (LWG) in kg.
[3]Van Amburgh and Drackley (2005), RE = (0.11 × $EBWG^{1.1684}$ × $EBW^{0.75}$) × 1000.
[4]Proposed, RE = ($EBWG^{1.0285}$ × $EBW^{0.21}$) × 1000.
Empty body weight (EBW) and daily EBW gain are in kg.

An equation to predict RE, considered equivalent to the net energy required to support the observed growth ($_{req}\mathbf{NE_g}$), was also developed using EBW and EBWG as the input and utilizing a non-linear model (Adj. R^2 = 0.89, RMSE = 0.23):

$$RE = EBW^{0.212 \pm 0.005} \times EBWG^{1.029 \pm 0.04} \quad [1]$$

where RE is in Mcal per day, EBW is in kg and EBWG in kg per day.

This equation was compared with that from Toullec (1989), adopted by NRC (2001), and that proposed by Van Amburgh and Drackley (2005). Measurements of model adequacy are in Table 1. As previously observed by Van Amburgh and Drackley (2005), the equation from Toullec (1989) over-predicted RE because it was developed for heavy veal calves fed high fat diets. The accuracy of RE prediction was improved by the use of the equation from Van Amburgh and Drackley (2005), however mean and slope biases were still observed ($P < 0.001$). Equation [1] provided the best performance with no bias and high CCC. The proposed model allows for the evaluation of energy balance on a net basis by comparing the RE allowable by the diet (MEg × $_{var}k_g$) with the predicted $_{req}NE_g$ with equation [1].

Nitrogen and Amino Acid Supply and Requirements

Net N Requirements

Non-productive N losses were considered to be endogenous N (EN) that is lost in feces, urine and scurf. Daily scurf and urinary N losses were estimated as 0.035 g N/kg$^{0.60}$ full BW and 0.44 g N/d per kg$^{0.50}$ of full BW, respectively (Swanson, 1977). By regressing the apparently digested N against the ingested N, both expressed as a fraction of DMI, endogenous fecal N losses (EFN = 3.53 ± 0.81 g/kg DMI) and true digestibility of dietary N from milk proteins (0.99 ± 0.02) were estimated.

A predictive equation for RN, as an estimate of the net N requirements for growth ($_{req}NN_g$) was derived by simple linear regression using individual daily EBWG as explanatory variable (RSME = 2.28):

$$RN\ (g/d) = 1.31 \pm 0.62 + 28.29 \pm 0.94 \times EBWG\ (kg/g) \quad [2]$$

In this equation, both intercept and slope were significant (P < 0.04), and the prediction accounted for 92% of the variation in actual RN, with no mean (P = 1.00) or slope bias (P = 0.69). Davis and Drackley (1998) proposed to calculate RN from ADG using a fixed coefficient of 30 g N/kg ADG, and NRC (2001) adopted it. Such an approach agrees with the coefficient obtained here (29.96 ± 0.52) when RN was regressed as a function of EBWG with no intercept. However, predictions using the approach showed mean (P = 0.02) and slope bias (P = 0.03). In their review Davis and Drackley (1998) reported that N in the gain was not constant, which is in agreement with the present data set, where it ranges from 23.67 to 54.83 g N/kg EBWG. Although a subtle change, the inclusion of the intercept in the relationship is powerful as it helps to better predict this variation in N content of gain and allows for the representation of decreasing N concentration in the gain as EBWG increases.

Thus, after computing the EN lost in scurf, feces and urine, in g/d, they were aggregated to calculate the net non-productive (i.e. maintenance) N requirements ($_{req}NN_m$). These were added to the RN to represent the total daily net N requirement of the calf ($_{req}$NN, g/d)

Metabolizable N supply

Extrapolating the assumption made for swine, that the contribution of EN from the large intestine is 10% of the EFN (NRC, 2012), the estimated EFN was related to basal ileal EN losses (EFN × 0.9). Also, based on the data from Montagne et al. (2000), the amount of EN at the terminal ileum was 30% of that flowing at the jejunum, and assuming this latter pool closely represented the EN generated at the foregut and midgut, the reabsorbed EN was calculated as EFN × 2.1. Ingested N was multiplied by the true digestibility to estimate the absorbed N from the diet, and that together with the reabsorbed N from endogenous origin constituted the metabolic N supply (MNS) for the calf.

Efficiency of Metabolizable N and AA Utilization

In order to model N and AA balance, the different N pools were associated to an AA profile (Molano, 2020). Calculations were made considering AA in their hydrated state, accounting for the molecule of water added to each AA after protein hydrolysis. The AA profile of EN used in the digestive process was assumed to be equivalent to that of the EN flowing at the terminal ileum, which for the most part was adopted from Montagne et al. (2000), while that of Phe, Trp and Tyr were obtained from Gerrits et al. (1997). The AA lost in scurf corresponded to the AA profile of the hide (Gerrits et al, 1997). Milk replacers were analysed for AA at Cornell University following the procedure described by Van Amburgh et al. (2015). These profiles were applied to the truly digested feed N to calculate the absorbed AA. And because these profiles were determined using 24-h acid hydrolysis and this procedure has been shown to not ensure complete AA recovery (Fessenden et al., 2017; Lapierre et al., 2019), correction factors generated by Ortega et al. (unpublished) for animal tissues and milk protein were applied to the reported AA profiles of endogenous and milk replacer, respectively. Body fractions and whole milk AA profile were determined by Ortega et al. (unpublished) using multiple time hydrolysis and estimating the concentration of AA bonded as protein before hydrolysis for each matrix using nonlinear regression. Hydroxyproline content of the body fractions were adopted from Williams (1978).

A combined efficiency of use of absorbed N (k_N) and AA (k_{AA}) was defined as the ratio between the described requirements, both productive and non-productive, and the metabolizable supply ($_{req}$NN/MNS and $_{req}$NAA/MAAS, respectively). Considering that the in the calf the chemical properties of nutrients consumed in the liquid feed are conserved during digestion and closely mirror those ultimately available for metabolism, k_N and k_{AA} were regressed against the metabolizable supplies relative to total ME, or to the ME associated to the dietary fat, CP or carbohydrate alone. Nitrogen and AA efficiency of use has been related to their respective supplies relative to energy in swine (Kyriazakis and Emmans, 1992) and lactating dairy cows (Van Straalen et al., 1994; Higgs and Van Amburgh, 2016). This relationship allowed the use of a variable efficiency of use that is considered to be biologically sound and improves model predictions, when compared with the use of constant coefficients (Van Straaleen et al., 1994; Nozière et al., 2018; Lapierre et al., 2020).

The N or AA supply relative to ME were inversely related to their combined efficiency of use, in agreement with the diminishing return law, supporting the idea that energy is the primary driver of protein synthesis and efficiency of AA use is energy dependent (Miller, 2004). However, in the pre-ruminant calf, the relationship between the efficiency of use and the supply relative to total ME, was not conserved when ME was partitioned into the nutrients generating the energy. This indicated that at a given AA supply the efficiency of AA utilization was more closely related to the intake of carbohydrates, than to that of fat or protein calories per se. Although AA supply (particularly Leu) stimulate protein synthesis (Suryawan and Davis, 2011), the association between k_{AA} and protein intake, or its AA profile, was weak in agreement with previous analysis in both ruminants and non-ruminants (Miller, 2004; Higgs and Van Amburgh, 2016). Further, in veal calves Roy et al. (1970) concluded that fat was not a suitable energy source to increase N retention. In agreement with this observation, van den Borne

et al. (2007) noted that urea production, an indicator of protein breakdown, increased when more fat in the milk replacer was fed to veal calves. Further, using stable isotopes, van den Borne et al., (2007) were unable to find any carbohydrate carbon in adipose tissue, suggesting the carbohydrate was being preferentially used for functions like protein synthesis, which is consistent with the behavior in urea N levels.

The stronger relationship between carbohydrate energy and AA efficiency of use observed in this analysis is supported by findings in growing pigs where N retention was improved by increasing dietary starch at various levels of protein intake (Fuller and Crofts, 1977). In a similar manner, milk protein yield has been increased by the post-ruminal infusion of glucose in dairy cows (Rulquin et al., 2004). The major mechanism involved in the better utilization of N and AA through increased carbohydrate intake has been attributed to changes in insulin and its downstream signaling pathway related to protein synthesis (Fuller et al, 1977). In the short term, insulin rapidly activates protein synthesis by activating components of the translational machinery, while in the long-term, insulin also increases the cellular content of ribosomes to augment the capacity for protein synthesis (Proud, 2006). Insulin could also stimulate muscle protein synthesis by increasing the efficiency of translation (Davis et al., 2001). The relationship between the treatment mean combined efficiency of utilization and the supply for N and AA relative to carbohydrate ME was best described using a loglogistic model with three parameters. Parameters estimates and fit summary from this regression are presented in Table 2.

Similar to the approach proposed for energy, N balance could be assessed in net terms comparing ${}_{req}NN_g$ (equation [2]) versus allowable RN [RN (g/d) = (MNS (g/d) × k_N) – ${}_{req}NN_m$ (g/d)] using the combined efficiency estimated based on the grams of N per Mcal of carbohydrate ME provided from the diet. This same approach could be applied to the calculation of AA requirements.

An example of the relationship between efficiency of use of total AA and their supply relative to total ME and carbohydrate ME with the fit of the loglogistic function is illustrated in Figure 2.

Table 2. Model parameters and fit summary of the loglogistic regression between the combined efficiency of use and supply of N and AA relative to carbohydrate ME.

Item	Log logistic Model Parameters[1]			RMSE	Adj. R^2
	$\boldsymbol{\theta}_1$	$\boldsymbol{\theta}_2$	$\boldsymbol{\theta}_3$		
N	1.04	1.92	42.97	0.04	0.88
Arg	2.06	2.02	8.50	0.10	0.85
His	1.25	1.66	5.16	0.05	0.88
Ile	0.76	0.95	8.58	0.02	0.91
Leu	3.39	0.60	0.82	0.03	0.88
Lys	0.87	1.30	16.36	0.03	0.90
Met	1.15	1.79	4.38	0.06	0.78
Met+Cys	0.69	3.01	11.67	0.06	0.67
Phe	1.30	1.56	8.08	0.04	0.93
Phe+Tyr	1.20	1.92	15.73	0.05	0.91
Thr	0.80	1.68	14.21	0.03	0.90
Trp	2.92	0.75	0.41	0.03	0.95
Val	0.83	1.48	14.68	0.03	0.87
Total AA[2]	1.26	1.24	186.35	0.04	0.87

[1]$k_{(N \text{ or } AA)} = \boldsymbol{\theta}_1/(1+\exp(\boldsymbol{\theta}_2 \times \text{RM(N or AA)S} + \log(\boldsymbol{\theta}_3)))$, where RM(N or AA)S is the metabolizable supply of N or AA relative to carbohydrate ME (g/Mcal).
[2]Total AA = EAA and NEAA. All AA expressed as hydrated residues

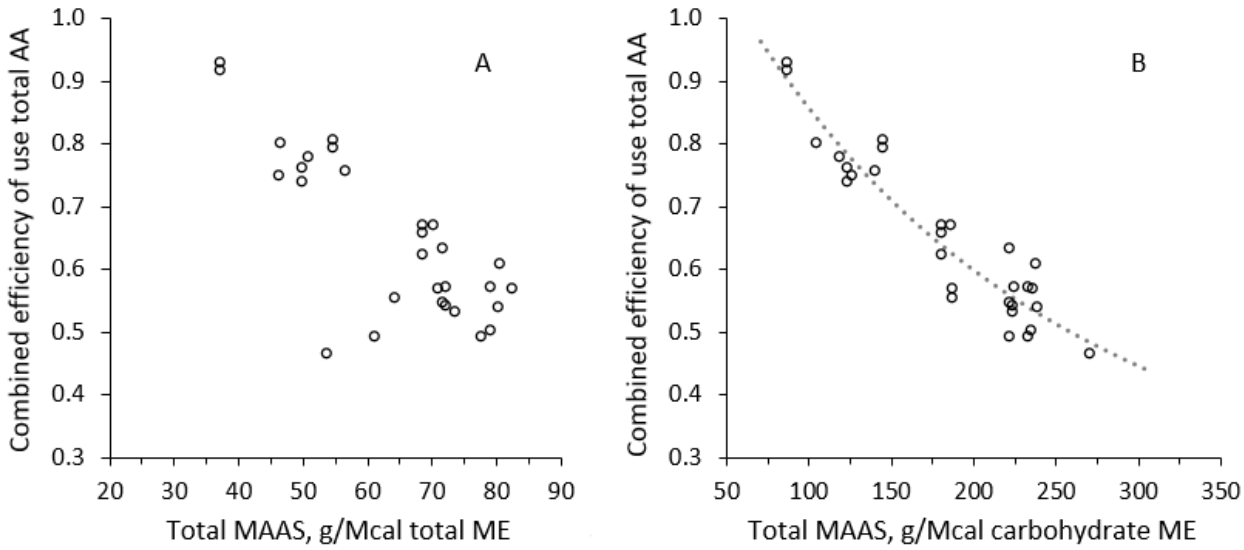

Figure 2. Relationship between combined efficiency of use and metabolizable supply of total AA (EAA and NEAA) relative that of total ME (A) or ME from carbohydrates fitted by a linear and loglogistic model (dotted line, B). Circles represent treatment means.

Application

In order to integrate the concepts and results disused in the present analysis, estimation of energy and total AA requirements per the proposed equations were calculated for Holstein calves of three different live BW (50, 65, and 80 kg) growing at different rates of daily weight gain.

Energy requirements for growth were calculated for the EBW and EBWG and are presented in Table 3. Metabolizable energy requirements for maintenance, assuming thermo-neutrality, increased with BW indicating that as calves grow, more calories are needed to maintain life only, and therefore feeding programs should adjust to provide enough calories above maintenance to achieve targeted gains. As BW increases from 50 to 80 kg, the increment on ME maintenance requirements would correspond to approximately 150 g of DM of milk or milk replacer per day. Required NE_g increased with rate of gain at a given BW, and with BW for a given gain since energy content of the gain increases as the animal matures. Because energy requirements relative to BW also increase with rate of gain, the efficiency of use of ME for growth (k_g) decreases. In contrast, k_g increased with calf size at a given rate of gain, because the energy required relative to BW decreases. Using the predicted k_g, growth requirements were converted on a metabolizable basis, which follow a similar pattern as that described for NE_g.

Table 3. Effect of varying live BW and rate of gain of milk-fed Holstein calves under thermo-neutral conditions on the estimation of energy requirements and utilization for growth according to the proposed model.

Live BW, kg	Live BW gain, kg/d	ME_m[1], Mcal/d	NE_g[2], Mcal/d	k_g[3]	ME_g[4], Mcal/d
50	0.2	1.8	0.4	0.74	0.5
	0.4		0.8	0.66	1.2
	0.6		1.2	0.60	2.0
	0.8		1.6	0.56	2.9
65	0.4	2.2	0.9	0.67	1.3
	0.6		1.3	0.62	2.1
	0.8		1.7	0.58	3.0
	1.0		2.2	0.55	4.0
80	0.6	2.5	1.4	0.64	2.1
	0.8		1.8	0.60	3.0
	1.0		2.3	0.56	4.1
	1.2		2.8	0.54	5.1

[1] $0.1 \times EBW^{0.75}$

[2] $EBW^{0.212} \times EBWG^{1.029}$

where: EBW (kg) = live BW × 0.93; EBWG (kg/d) = live BW gain × 0.92

[3] Calculated iteratively as $k_g = 1/(1 + \exp(0.46 \times (\log(ME_g) - 5.64)))$

Where MEg = ME available for gain (kcal/$kg^{0.75}$ of EBW)

[4] NE_g / k_g

Using the estimated EBW, EBWG, total ME requirements and DMI as inputs, net N requirements for maintenance and growth requirements were calculated using the proposed equations and transformed to total AA (Table 4) using the total AA N in each pool and the N content of the total hydrated AA (Molano, 2020). Net requirements for maintenance increased slightly with BW at a given rate of gain, while net requirements for both maintenance and growth increased in a greater magnitude with rate of gain, due to the greater metabolic endogenous AA secreted with increased DMI and to the greater need for AA to support tissue deposition. This indicates that AA requirements of the calf are mainly a function of rate of growth and not BW, which agrees with the observations made by Davis and Drackley (1998) for apparently digestible protein requirements. Thus, as proportion of the total net requirements of total AA, maintenance requirements are diluted as rate of gain increases, accounting for nearly half of the total requirements for a 50 kg calf growing at 0.20 kg/d while just about a fifth of the total requirements when growing at 0.80 kg/d.

In order to convert the net requirements of total AA to metabolizable basis the efficiency of use was estimated iteratively assuming the milk replacer being consumed was 43% carbohydrates and 6% ash. Overall, the combined efficiency of use decreased as rate of gain increased but, increased with BW at a given rate of gain, following the relative contribution of maintenance requirements to the total net requirements described earlier. Further, this indicates that total AA are used with a higher efficiency for maintenance than for growth functions. Subsequently, total AA supplied from metabolic endogenous reabsorption were estimated and subtracted from the total metabolizable requirement to calculate the total metabolizable AA needed to be supplied by the diet. In addition, because in the proposed model AA requirements and supplies are expressed as hydrated residues resulting from protein hydrolysis (free AA), the estimated total dietary metabolizable AA supply was multiplied by 0.86 to correct their molecular weight to anhydrous basis (Lapierre et al., 2016), which is the from AA are bonded in protein, and be able to represent the metabolizable protein required in the diet. Considering the estimated true digestibility of milk proteins (0.99), the dietary metabolizable protein supply was converted to true protein intake and then expressed as a percentage of the DM based on the estimated DMI. The calculated concentration of true protein in the milk replacer increased with the rate of gain and decreased as BW increased to achieve a given rate of gain. Estimated true protein content of the diet for the lighter calves growing at the fastest rate was identical to that of whole milk (25% of DM), and corresponded closely to the crude protein requirement previously reported by Van Amburgh and Drackley (2005; 26 to 28% of DM). Also, these calculations suggest that the true protein content of the diet required to support a given rate of gain is not static and decreases as calves matures, which is in agreement with the analysis made by Davis and Drackley (1998). This observation of decreasing TP is primarily due to the increase in ME requirements, which include maintenance, and the increase in DMI which are both greater than the increase metabolizable protein requirements, resulting in a dilution of the required true protein in the DM. As described here for total AA, the calf's requirements and supplies of particular AA could be estimated following this methodology.

Table 4. Effect of varying live BW and rate of gain of milk-fed Holstein calves under thermo-neutral conditions on the estimated net and metabolizable total AA and dietary true protein requirements according to the proposed model.

Live BW, kg	Live BW gain, kg/d	Total ME required, Mcal/d	DMI[1], kg/d	Total AA Requirements					Dietary true protein	
				Net requirements, g/d		k_{TAA}[4]	Metabolizable requirements, g/d		Metabolizable supply[6], g/d	Percent in DM[7]
				Maintenance[2]	Total[3]		Total	Diet[5]		
50	0.2	2.3	0.4	28	69	0.74	94	80	68	16
	0.4	3.0	0.6	31	105	0.63	165	147	125	21
	0.6	3.8	0.7	33	140	0.59	237	213	182	24
	0.8	4.7	0.9	36	176	0.58	304	274	233	25
65	0.4	3.4	0.7	35	109	0.70	156	135	115	18
	0.6	4.2	0.8	37	144	0.65	222	196	167	20
	0.8	5.2	1.0	40	180	0.63	286	254	216	22
	1.0	6.2	1.2	43	216	0.62	345	307	262	22
80	0.6	4.7	0.9	41	148	0.70	213	184	157	18
	0.8	5.6	1.1	44	184	0.67	274	239	204	19
	1.0	6.6	1.3	47	219	0.66	332	291	248	20
	1.2	7.7	1.5	50	255	0.66	386	339	289	20

[1]Based on milk replacer with 43% carbohydrate, 6 % ash and ME content between 5.1 and 5.4 Mcal/kg of DM.
[2]Calculated from the net N maintenance requirements (EFN, scurf N and EUN) × Total AA N in N of each pool / AA N content of each pool.
where: EFN (g/d) = DMI (kg/d) × 3.53; Scurf N (g/d) = 0.035 × $BW^{0.6}$; EUN (g/d) = 0.44 × $BW^{0.5}$. BW (kg) = live BW.
[3]Total AA net requirements = maintenance ([3]) + growth (Retained N × Total AA N in EBW N / Total AA N content)
where: Retained N (g/d) = 1.31 + EBWG × 29.28; EBWG (kg/d) = live BW gain × 0.92.
[4]k_{TAA} = Combined efficiency of total AA use, calculated iteratively as 1.26/(1+exp(1.24 × RMTAAS+ log(186.35))), setting 0.58 as starting point and assuming a milk replacer with 43% carbohydrate and 6% ash (DM basis).
where RMTTAS = metabolizable total AA supply relative to ME intake from carbohydrate (g/Mcal ME).
[5]Metabolizable total AA required from the diet = total metabolizable requirement (total net requirements / k_{TAA}) - metabolizable total AA supply from endogenous origin [(Reabsorbed EN, g/d = DMI (kg/d) × 3.53 × 2.1) × Total AA N in endogenous N / Total AA N content].
[6]Dietary metabolizable protein = dietary metabolizable total AA × 0.86, to convert hydrated AA to anhydrous basis.
[7]Considering milk-protein true digestibility of 99%, true protein content (% DM) = (Metabolizable protein / 0.99)/DMI (g/d) × 100.

Summary

Using the body composition data available, a set of equations were developed to estimate energy and AA requirements for the pre-ruminant calf. Likewise, this analysis allowed us to describe nutrient utilization using a variable partial efficiency of ME use for growth, calculated as a function of feeding level, and a variable combined efficiency of N and AA use determined based on their relative supply to carbohydrate ME. As a whole, the proposed model offers a mechanistic approach to estimate energy and AA requirements on a net basis and allows the user to evaluate their balance.

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Gut Health Challenges: How Do We Feed to Improve Intestinal Integrity and Growth In Calves?

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Introduction

During the preweaning and weaning periods for calves, there is heightened susceptibility to disease and gastrointestinal dysfunction, specifically in the small intestine before maturation of the rumen (Steele et al., 2016). Morbidity and mortality related to diarrhea and other digestive issues continue to be an issue in young calves (Urie et al., 2018). Diarrhea in young calves reduces dry matter intake, body weight gain, and feed efficiency (Morrison et al., 2019). Understanding feeding practices that promote improved health and productivity of dairy replacement animals is critical for future success in the herd.

While nutritional focus in mature cattle is centered on the rumen, shifting focus to the small intestine in the preweaning period is essential and necessary to optimize gastrointestinal growth potential, while also minimizing the risk of enteric challenge. Gut growth is regulated by several factors, including metabolic and trophic hormones, and chemical and physical properties of the diet (Baldwin et al., 2004). The interaction of the gut mucosa, microbiota, and feed is complicated (Niewold, 2015). As we further develop our understanding of the gastrointestinal tract's (GIT) impact on calf health and growth, we can promote feeding strategies to optimize development and integrity of the GIT as well as minimize enteric challenges.

Incidence Rate and Outcome of Diarrhea in Calves

Incidence rate of morbidity and mortality continue to affect a large proportion of calves in the United States and around the globe. Survey information of morbidity and mortality rates of heifers collected in the United States in 2014 was 33.9% and 5.0%, respectively (Urie et al. 2018). Of the cases recorded, 56.0% of morbidity and 32.0% of mortality cases in heifer calves were attributed to digestive signs (Urie et al., 2018). The highest rate of abnormal feces is commonly seen within the first 3 wk of life (Bartels et al., 2010) with the greatest risk of treatment for diarrhea around 10 d of age (Waltner-Towes et al., 1986; Windeyer et al., 2014).

Calves may be predisposed to developing diarrhea in the first 21 d of life if they have increased intestinal permeability at birth (Araujo et al., 2015). Additionally, calves that are given a delayed colostrum feeding have greater paracellular permeability, which could indicate slower tight junction closure that could allow pathogenic bacteria to further disrupt intestinal permeability and could result in diarrhea (Araujo et al., 2015). Several studies in calves have indicated higher intestinal permeability in the second week of life

(Araujo et al., 2015; Morrison et al., 2017) which correspond to increased fecal scores and could indicate damage to the villi in the small intestine (Hall, 1999).

Animals that undergo either clinical or subclinical infections will eat and grow less and overall have reduced efficiency (Johnson, 1998). A dataset created from four experiments of transported calves classified them as either healthy or diarrheic in the first 21 d after arrival (Morrison et al., 2019). A retrospective analysis of health status was conducted to determine intake and growth of the calves which were managed similarly. In total data from 313 calves were used in the analysis with 96 calves classified as diarrheic [fecal score >2 (scale 1-4) for ≥3 d in the first 21 d after arrival]. Intake of milk replacer, water, starter, and electrolytes were all recorded. Body weight and growth were also measured.

The cumulative number of days with elevated fecal scores were 1.88 vs. 6.84 ± 1.19 d for healthy and diarrheic calves, respectively. Initial total protein concentrations were not different between classifications. Intake of milk replacer for calves classified as diarrheic was lower and those calves were more likely to refuse part of the offered milk replacer amount. Intake of electrolytes was greater for calves classified as diarrheic. Cumulative starter intake was 40% lower in calves that were classified as diarrheic (0.9 kg) compared with calves that were healthy (1.5 kg) in the first 21 d after arrival. While starter intake does not make up a large portion of intake in this early preweaning period, the impact of diarrhea was evident. Although not measured in this study and the timeframe was fairly short, lower starter intake resulting from a diarrheic event could delay rumen development if this pattern of reduced starter intake continued. Finally, calves that were diarrheic had a 27% reduction in average daily gain (491 vs. 669 g/d), lower stature growth, and were less efficient (0.56 vs. 0.77 kg/kg; Morrison et al., 2019).

Longevity and productivity of the cow have been associated with events in the calf period. Specifically, calves treated with antibiotics have decreased lifetime milk production (Soberon et al., 2012) and the number of days in the first 4 mo of life that a calf is sick negatively impacts first-lactation 305-d metabolizable energy and actual milk, protein, and fat production (Heinrichs and Heinrichs, 2011). Further work is needed to continue to minimize the effect of digestive illness to improve production and welfare, and reduce increased costs associated with this issue.

Interaction between the Calf Gastrointestinal Tract, Feed, and Microbiota

The GIT of the animal, feed, and microbiota interact to form a dynamically complex ecosystem that when in balance work to support the health and growth of the animal (Niewold, 2015). The GIT is a barrier that is able to selectively discriminate the contents of the lumen to allow selective absorption of nutrients, while also providing a protective barrier to harmful antigens and pathogens (Groschwitz and Hogan, 2009). A mixture of different epithelial cells in the GIT form a physical and biochemical barrier to separate the luminal contents and microorganisms from the host mucosa and immune system to maintain coexistence (Peterson and Artis, 2014).

Intestinal Structure and Cells

The small intestine is composed of absorptive epithelial cells (enterocytes), nerve cells, goblet cells, immune cells, and enteroendocrine cells (Peterson and Artis, 2014; Niewold, 2015). Mature enterocytes work through active and passive transport and brush border enzyme activity to absorb nutrients (McOrist and Corona-Barrera, 2015). The enteric nervous system is important for motility, secretion, blood flow, and the immune system (Hansen, 2003).

A physical barrier is formed with the production of mucus from goblet cells, antimicrobial peptides, and immunoglobulin A (IgA; Hooper and Macpherson, 2010) which are important sites for both innate and adaptive immunity (Turner, 2009). The mucus layer is a first line of defense against bacterial translocation to the mucosa while continuing to allow nutrients to be transported across the mucosa (Atuma et al., 2001; Kim and Ho, 2010). Antimicrobial peptides have different actions but many target the cell wall or membrane, while others enzymatically attack cell structures (Gallo and Hooper, 2012; Hooper and Macpherson, 2010). Intestinal epithelial cells secrete IgA antibodies that help regulate commensal bacteria by limiting bacterial association with the intestinal epithelial surface (Hooper and Macpherson, 2010; Peterson and Artis, 2014).

Enteroendocrine cells represent approximately 1% of epithelial cells in the intestine and link central and enteric neuroendocrine systems through hormone regulators of digestive function (Peterson and Artis, 2014). Biological functions regulated by gut peptides include food intake, gastric emptying, motility, barrier function, and glucose metabolism. Therefore, gut peptides secreted from enteroendocrine cells play an important part in absorption of nutrients but also maintenance of barrier function (Cani et al., 2013).

Intestinal Permeability

Permeability of the GIT is location dependent (Penner et al., 2014) and changes with age (Wood et al., 2015). Transcellular permeability is responsible for the transport of solutes, including amino acids, electrolytes, short-chain fatty acids, and sugars, through selective transporters (Groschwitz and Hogan, 2009). Paracellular permeability is the transport of molecules through the space between the epithelial cells via the apical-lateral membrane junction and the lateral membrane (Van Itallie and Anderson, 2006). Expression of junctional proteins are dependent on location within the intestine, location on the microvilli, and location between epithelial cell membranes (Groschwitz and Hogan, 2009). In ruminants, permeability of passive ions is greatest in the jejunum and least for the rumen and omasum (Penner et al., 2014). Furthermore, small pore permeability increased after the rumen and omasum until the jejunum and then decreased in the ileum (Penner et al., 2014). Small intestinal permeability can be measured non-invasively by dosing two different sized non-digestible probe molecules (Hall, 1999; Menzies et al., 1979; Uil et al., 1997). The larger molecules indicate paracellular permeability while the smaller molecules indicate transcellular permeability (Bjarnason et al., 1995).

Inflammatory Response

The mucosal immune system works to tolerate contents and microorganisms in the lumen and is activated when foreign antigens translocate the GIT barrier (Niewold, 2015). The recruitment of circulating inflammatory cells occurs with increased production and secretion of pro-inflammatory cytokines in response to foreign antigens (Al-Sadi et al., 2009). Pro- and anti-inflammatory cytokines regulate intestinal barrier function differently (Al-Sadi et al., 2009). An increase in pro-inflammatory cytokines increases the disruption of the tight junction barrier and overall increases GIT permeability (Al-Sadi et al., 2009; Ma and Anderson, 2006; Nusrat et al., 2000; Bruewer et al., 2006; Shen and Turner, 2006). Alternatively, anti-inflammatory cytokines counteract some inflammation to help maintain tight junction functionality (Madsen et al., 1997; Forsyth et al., 2007).

Trophic Hormones and Peptides

Cells within the GIT secrete a number of hormones and peptides that signal maintenance, growth, and repair of epithelial tissue (Drucker et al., 1994; Burrin et al., 2003). One of particular interest and research in recent years is glucagon-like peptide 2 (GLP-2) which has a role in influencing trophic and regenerative actions in the intestinal epithelium (Burrin et al., 2000). Upon ingestion of nutrients, specifically carbohydrates and lipids, GLP-2 is secreted from the intestinal L-cells along the jejunum, ileum, and colon (Estall and Drucker, 2006; Larsson et al., 1975; Eissele et al., 1992). Specifically, GLP-2 has been shown to increase crypt cell proliferation and reduce apoptotic cell numbers which increases small intestinal mass (Tsai et al., 1997; Drucker et al., 1997). Furthermore, reductions in intestinal inflammation and increases in nutrient absorption in response to GLP-2 have been observed (Furness et al., 2013; Sigalet et al., 2007; Brubaker et al., 1997; Shirazi-Beechey et al., 2011).

Overall, factors that regulate gut growth include metabolic and trophic hormones, and chemical and physical properties of the diet (Baldwin et al., 2004). There are large energetic and nutrient costs associated with maintenance of the GIT in animals that are growing which greatly influences whole body metabolism (Baldwin et al., 2004). However, the actual energetic and nutrient cost is complicated by the influence of changes in tissue mass in response to plane of nutrition, chemical composition of the diet, and physiological status of the animal (Baldwin et al., 2004).

Intestinal Dysfunction

There are several instances that can lead to intestinal dysfunction, including pathogenic and nutritional insults that negatively affect intake, growth, and efficiency. Dysfunction of the GIT can be classified into three categories: 1) mucosal barrier disruption, 2) altered motility, and 3) atrophy of the mucosa (Martindale et al., 2013). All of these effects have been associated with enteric disease attributed with pathogenic bacteria resulting in diarrhea (Connor et al., 2013, 2017; Walker et al., 2015) and weaning (Malmuthuge et al., 2013; Eckert et al., 2015; Wood et al., 2015).

As the intestinal barrier becomes dysfunctional, an increased risk of foreign antigens and harmful bacteria accessing the underlying mucosa can lead to increased inflammation in the intestine (Cameron and Perdue, 2005). Under these conditions, the adaptive immune system is activated which reallocates resources previously utilized for growth to the production of immune cells and antibodies (Iseri and Klasing, 2013). Reduced appetence and catabolism of muscle resulting in a reduction in growth is a consequence of increased inflammation, which further increases susceptibility to intestinal pathogens (Niewold, 2015). Actions of enteric pathogens, including viruses, bacteria and protozoa, vary and affect different locations in the GIT. Damage caused by enteric pathogens can include intestinal villus and colonic crypt atrophy, secretion of enterotoxins, necrosis, and disruption of epithelial tight junction (Cho and Yoon, 2014; Foster and Smith, 2009). Damage caused in the GIT can cause prolonged malnutrition and result in decreased growth rates (Cho and Yoon, 2014).

Nutrient induced secretion of GLP-2 and the associated effects in pig models has been suggested as an important element in intestinal adaptation during neonatal phases by improving mucosal cell proliferation, barrier function, and the inflammatory response (Burrin et al., 2003; Cameron and Perdue, 2005; Sigalet et al., 2007; Ipharraguerre et al., 2013). Since GLP-2 secretion is responsive to nutrient intake, circulating GLP-2 is reduced when milk ingestion drops below 0.875% of calf body weight on a DM basis (Castro et al., 2016). Understanding and promoting GLP-2 and other trophic hormones could be important targets for improvements in intestinal integrity in situations that reduce feed intake like incidences of diarrhea or weaning (Connor et al., 2016). Additional information on nutrient and ingredient influence of motility could also aid in preventing and recovering from intestinal dysfunction in calves.

Lower feed intake can lead to reduced growth and development of the intestinal mucosa (Buchman et al., 1995; Groos et al., 1996). In a piglet model, varying levels of intake were fed to evaluate the amount of intake required to normalize intestinal growth (Burrin et al., 2000). In this study, the authors observed that the proximal segments of the small intestine were most sensitive and that 40% of total nutrient intake was needed to increase wet weight and protein content, while the ileum requires 60% of enteral intake however, 80% of total intake was required to normalize wet weight and protein content in both sections (Burrin et al., 2000).

Feeding and Diet Considerations

While colostrum has critical importance in terms of nutrients and bioactive factors (Blum and Baumrucker, 2008; Nissen et al., 2017) and weaning strategies impact on GIT development and function, the focus for this will center on feeding strategies and diet considerations in the preweaning period. Obviously, the transition into the ruminant phase and ruminal development continues to be a priority in terms of long-term animal success within the herd but areas of opportunity for improvement in intestinal dysfunction contributing to morbidity and mortality in the preweaning period are important.

Feeding Rate and Intake

Enhanced feeding rates of 20% of body weight, which are close to ad libitum intake, have been linked to increased body weight and growth, organ development and growth, metabolic and endocrine changes, improved feeding behavior, and immune and health (Hammon et al., 2020). Increased GIT growth rate and protein accretion of calves with enhanced feeding have been observed when calves are fed whole milk or milk replacer in comparison to calves fed 4 to 6 L/d (Geiger et al., 2016; Schäff et al., 2016; Korst et al., 2017). If you consider a 50 kg calf that is fed 20% of its body weight as milk or milk replacer, the calf would be offered 10 L per day. In contrast, the same calf only fed 4 or 6 L/d would be only 40 to 60% of the enhanced feeding rate. In neonatal piglets, 40 to 60% of normal intake reduces small intestinal mass and protein content, while 80% of intake was needed to normalize this (Burrin et al., 2000). Decreased circulating GLP-2 concentrations at similar reduced intake has been observed when intake drops below 0.875% of body weight as DM indicating lower trophic actions in the gut (Castro et al., 2016).

These changes in intestinal growth would be in line with observed increases in organ growth, including the small intestine, in response to increased feeding levels in calves (Geiger et al., 2016; Koch et al., 2019). Furthermore, increased surface area and absorptive capacity in the small intestine results from increased feeding rates (Geiger et al., 2016; Koch et al., 2019). Intestinal growth was likely mediated by changes in the local IGF system (Ontsouka et al., 2016). Concerns over delayed rumen development because of delayed starter consumption (Khan et al., 2011) are common with higher levels of milk or milk replacer intake but comparable rumen development and transition can be achieved when an appropriate weaning timeline is used (Schäff et al., 2018).

Greater nutrient supply has been suggested to improve intestinal maturation by supporting a proper adaptive immune response and stabilizing microbiota within the GIT to minimize risk of enteric challenges preweaning (Hammon et al., 2020). Adequate nutrient supply may be required to mature the GIT immune system and to be able to defend against invasive enteric pathogens (Khan et al., 2011; Hammon et al., 2018). Increased feeding rate, and therefore energy intake with higher fat and protein, can result in faster improvement of fecal scores as a result of an infection with *Cryptosporidium parvum* (Ollivett et al., 2012). This may be a result of enhanced activation of the intestinal immune system (Hammon et al., 2018) and a better ability to resist infection (Ballou et al., 2015).

In addition to decreased milk allowance in the preweaning period, reductions in milk and starter intake during an enteric challenge, like diarrhea, may contribute to intestinal atrophy commonly observed with many enteric pathogen infections. There has not been a lot of work specifically looking at level of intake after an enteric disease challenge and how this might help with recovery of GIT size and integrity. It is commonly suggested to not completely withdraw milk or milk replacer feeding when calves have diarrhea and to allow them to consume at least part of their nutrients through that source to aid in recovery (Garthwaite et al., 1994; Quigley et al., 2006; McGuirk, 2011).

Prolonged time without enteral intake of nutrients would likely result in protracted recovery of GIT function and health, but more work in this area is needed.

Dietary Characteristics

Specific dietary factors can impact GIT permeability and tight junction expression (Steele et al., 2016). Milk replacers often have higher content of lactose (42 to 45% DM vs. 35% DM) and lower content of fat compared with whole milk (Wilms et al., 2019). Differences in fat and lactose content change the energy density of milk replacers and influence the osmolality. Whole milk has an osmolality close to 300 mOsm/kg (McGuirk, 2003). While milk replacers have a range from slightly hypertonic (>300 mOsm/kg) to very hypertonic (>450 mOsm/kg; McGuirk, 2003; Wilms et al., 2019). Changes in osmolality in milk replacers can lead to disturbances of the GIT. A study evaluated GIT permeability in response to varying levels of osmolality (439 to 611 mOsm/kg) and replacement of lactose with monosaccharides (dextrose and galactose) in milk replacers observed that as osmolality increased GIT permeability increased (Wilms et al., 2019). Interestingly, osmolality and source of sugar did not impact growth, fecal DM, or fecal pH (Wilms et al., 2019).

Summary and Perspectives

Morbidity and mortality rates related to diarrhea and other digestive issues continue to be an issue in replacement programs. The GIT is a dynamic and complex system that changes throughout the preweaning period. Promoting development of the structural and metabolic actions of the GIT can improve calf growth while also minimizing intestinal challenges. Intestinal dysfunction, including pathogenic and nutritional insults can negatively affect intake, growth, and efficiency. By continuing to expand our understanding of normal development of the GIT, including the small intestine, we can either work to prevent intestinal dysfunction from occurring or target strategies for recovery after intestinal dysfunction has occurred.

Reduced intake of nutrients, either in normal feeding practices or illness, can lead to reduced GIT growth and permeability. Under these circumstances, actions of metabolic hormones like GLP-2 are reduced, which increases susceptibility to pathogenic microbiota. Other insults to intestinal permeability can include changes in osmolality. Further work with more specific types of ingredients or additives could also be useful in promoting GIT development and integrity.

If we can maximize intestinal integrity and balance so that intake is maximized in the preweaning period, the nutrients consumed by the calf can go toward GIT growth and not be used for increased maintenance costs of an infection. Furthermore, this will result in increased growth of the calf, optimal feed efficiency, rumen development, reduced medication costs, labor, and productive potential.

Take Away Messages

1. Enteric challenges resulting in morbidity and mortality of calves results in reduced efficiency.
2. The gastrointestinal tract is a complex system, but our understanding of its importance to calf development and health is expanding.
3. Feeding rate and nutrient provision positively impacts the growth and integrity of the gastrointestinal tract which can minimize risk of enteric disease.
4. Dietary characteristics of feeds could manipulate permeability.

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Organic Acid and Plant Botanical Supplementation in Heat-Stressed Holstein Calves

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Introduction

Dietary organic acid and plant botanical (OA/PB) supplementation represents a promising strategy to support and reduce antibiotic usage in livestock production systems. These natural compounds have unique antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory properties, which when combined, have potential to improve gastrointestinal health by controlling bacterial pathogen growth and enhancing barrier function. Organic acid and plant botanical feeding is a common practice on swine and poultry farms; however, these additives have received minimal attention for growing and lactating ruminants. This conference proceeding aims to review the fundamental concepts related to OA/PB feeding in domestic animals considering post-absorptive metabolism and intestinal health. The composition and properties of organic acids (i.e., citric and sorbic acids) and plant botanicals (i.e., thymol and vanillin) are summarized. The effects of OA/PB supplementation on feed intake and growth performance during heat stress conditions is discussed. A recent comprehensive study at Cornell University that investigated two levels of OA/PB supplementation in weaned and heat stressed Holstein calves and its effects on growth performance is presented in support.

Definitions and functions of organic acid and plant botanicals

Citric acid: A weak organic acid and intermediary metabolite of the citric acid cycle within mitochondria. It possesses antimicrobial properties. Its mode of action is proposed to involve the reduction in bacterial intracellular pH causing damage to enzymatic activity, protein, DNA, and extracellular membranes.

Sorbic acid: A short-chain unsaturated fatty acid that exerts antimicrobial and antifungal actions by inhibiting the microbial enzymatic apparatus and uncoupling the cell's nutrient transport system.

Thymol: A natural monoterpenoid phenol that has antioxidant properties, and promotes bactericidal activity and membrane permeabilizing actions towards pathogens such as *Salmonella enterica*.

Vanillin: A phenolic aldehyde widely used to increase palatability. The compound has anti-microbial activity but also anti-inflammatory and antioxidant potential.

Dietary organic acid and plant botanical supplementation: Lessons learned from swine and poultry production

The concept of feeding acidifiers such as citric and sorbic acids has been commonplace in swine and poultry production (Sofos et al., 1985; Roth and Kirchgessner, 1998; Partanen and Mroz, 1999). These compounds have also been utilized in the food industry for their protective effects against bacteria, fungus and mold (MacDonald and Reitmeier, 2017). As a feed additive, there is an extensive body of literature demonstrating their important role in maintaining gut health in livestock species. Their benefits and applications involve improving nutrient digestibility, enhancing immune function, exerting antimicrobial effects against pathogenic bacteria, and increasing growth performance (Pearlin et al., 2020). In swine production, an important feature of organic acid supplementation is the acidification of the digestive tract, especially for suckling animals. It has been shown that diet acidification for weaned piglets with 1% citric acid caused a reduction in stomach pH from 4.6 to 3.5 (Sciopioni et al., 1978). This pH control enables piglets to maintain an optimal pH for enzymatic action in the stomach and therefore improve protein digestion (Cranwell et al., 1976). The lowering of stomach pH may also restrict the growth of pH-sensitive pathogenic bacteria like *Escherichia coli (E. coli)*, *Salmonella* spp. and *Clostridium perfrigens*. The undissociated form of acidifiers can penetrate the bacterial cell, which possesses a neutral pH, and dissociate causing a reduction in intracellular pH, while inhibiting enzymatic reactions and nutrient transport (Mroz et al., 2006).

The *in vivo* effects of dietary OA/PB supplementation (25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride matrix) on intestinal integrity and inflammation of weaned pigs was also recently investigated (Grilli et al., 2015b). Dietary OA/PB supplementation promoted greater average daily gain and body weights of study pigs. The investigation also involved the collection of ileal and jejunal tissue samples post-weaning for Ussing chamber analysis of transepithelial electrical resistance, intermittent short-circuit current, and dextran flux. Results indicated that pigs fed OA/PB at 5 g/kg of body weight tended to have reduced intermittent short-circuit current in the ileum, which suggests improved intestinal barrier. These findings were supported by increased trans-epithelial resistance in Caco-2 cells grown in the presence of OA/PB (0.2 or 1 g/L; Grilli et al., 2015b). The authors were also able to demonstrate that feeding OA/PB downregulated the ileal gene expression of inflammatory cytokines including interleukin-12 and transforming growth factor-β) in pigs. This may mean that dietary OA/PB may ensure the integrity of the intestinal barrier by minimize inflammation. In a different study, Bonetti et al. (2020) demonstrated that sorbic acid and thymol reduce the growth of *E. coli* that express K88, the etiological agent of post-weaning diarrhea in pigs. Thymol also reduced the expression levels of *E. coli* K88 virulence genes. Lastly, Emami et al. (2017) supplemented *E. coli* K88-challenged broilers with three different organic acid mixtures. Organic acid therapy improved growth performance, ileal morphology, and primary and secondary immune responses, and increased cecal lactobacilli and reduced cecal *E. coli*.

In growing broiler chickens, Hassan et al. (2010) tested a 0.06% and 0.1% dietary supplementation level of two mixtures of organic acids (blends of fumaric acid, calcium format, calcium propionate, potassium sorbate, or citric acid, calcium formate, butyrate, calcium lactate, essential oils and flavoring compounds). Independent of mixture, birds supplemented with organic acids had increased body weight gain and feed conversion ratio compared to an unsupplemented control. Both organic acid mixtures also decreased intestinal counts for *E. coli* and *Salmonella* spp. Furthermore, Smulikowska et al. (2009) demonstrated that dietary organic acid supplementation with a blend of fumaric acid, calcium formate, calcium propionate and potassium sorbite at 1 g/kg of pelleted diet increased villi height, crypt depth, and the width of the tunica muscularis of broiler chicks. These histological outcomes are postulated to improve intestinal functionality by enhancing nutrient absorption to support growth.

Heat stress in Holstein dairy calves: Dietary organic acid and plant botanical supplementation to improve resilience

One of the greatest challenges facing dairy production in the United States is heat stress. It is estimated that ~$2.3 billion in economic losses are associated with decreased performance in heat-stressed gestating and lactating cows (St-Pierre et al., 2003; Ferreira et al., 2016). Growing dairy cattle also experience impaired growth performance that contributes to these economic losses. The physiological response to heat stress is characterized by decreased feed intake, increased sweating and respiration rates, and increased body temperature (Collier et al., 1982). These changes contribute to increases in maintenance energy costs that can range from 25 to 30% (Fox and Tylutki, 1998). Another important hallmark of heat adaptation in mammals is the redirection of blood supply from the visceral organs towards the body periphery (Hall et al., 1999). This causes ATP depletion, acidosis, altered ion pump activity, and oxidative stress in the intestinal epithelium (Hall et al., 1999, 2001). The insult provokes paracellular permeability and tight junction opening (Lambert, 2009), which may promote intestinal permeability and leakage of bacteria and their endotoxin into the circulation to stimulate local and systemic immune responses (Ghosh et al., 2020).

It is important to consider that growing animals experiencing heat stress not only possess an increased maintenance energy requirement but also experience decreased feed intake (Nonaka et al., 2008; O'Brien et al., 2010; Yazdi et al., 2016). In growing cattle, it appears that the reduction in feed intake accounts for the deficit in growth that occurs during heat exposure. O'Brien et al. (2010) determined that heat-stressed Holstein bull calves experienced a 12% reduction in feed intake during heat exposure (29.4 to 40°C for a period of 9 d), which completely accounted for the decrease in average daily gain. In a similar manner, Yazdi et al. (2016) demonstrated that lowered dry matter intake during heat exposure was the driver of lowered body weights in Holstein bull calves. However, it is important to note that in these previous studies, carcass composition was not evaluated, the effects of extended heat stress were not tested, and heifer calves were not studied. Moreover, the direct effects of heat stress on physiology and metabolism within the context of growth still deserves consideration in dairy calves. Although some of the post-absorptive metabolism changes in terms of increased circulating insulin levels

seem to be fairly similar between growing and lactating cattle (Rhoads et al., 2009; O'Brien et al., 2010), our understanding of the mechanisms and implications of heat-induced intestinal permeability in growing animals is still undeveloped. In addition, dietary therapies that enhance heat stress resilience in dairy calves need to be considered.

The ability of dietary citric and sorbic acids, thymol, and vanillin to enhance growth in heat-stressed calves has scientific merit. Using an *in vitro* approach, Grilli et al. (2015a) evaluated the effects dietary OA/PB supplementation on the growth of foodborne pathogens such as *E. coli* and *Salmonella typhimurium* using pure bacterial cultures as well as mixed ruminal microorganism fermentations. Several concentrations (i.e., 0.1, 0.5, 1.0, 2.0, 5.0, and 10.0% vol/vol) of water-solubilized OA/PB were tested. The results demonstrated that 2% OA/PB inclusion (relative to total culture volume) reduced pathogen growth rates. Pathogen populations were also reduced by OA/PB. These findings suggest that dietary organic acid and plant botanical supplementation may be a means to reduce potentially harmful populations of pathogenic bacteria found in the digestive tract of young dairy cattle experience heat stress. However, the effects of dietary OA/PB on growth in young calves was not yet defined.

Therefore, our lab completed a study which evaluated the effects of dietary OA/PB supplementation on growth performance in Holstein calves challenged by heat stress. In a completely randomized design, 62 bull and heifer calves were assigned to one of five groups (n = 11-14/group): thermoneutral conditions (TN-Con), HS conditions (HS-Con), thermoneutral conditions pair-fed to HS-Con (TN-PF), HS with low-dose microencapsulated OA/PB (75 mg/kg of body weight; 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride for rumen protection; AviPlus R; Vetagro, Italy; HS-Low), or HS with high-dose microencapsulated OA/PB (150 mg/kg of body weight; AviPlus R; HS-High). Supplements were delivered as a twice daily bolus via the esophagus wk 1 through 13 of life; all calves received boluses equivalent for triglyceride. Post weaning, calves (62 ± 2 d; 91 ± 10.9 kg) remained in thermoneutral conditions (temperature-humidity index [THI]: 60 to 69) for a 7-d covariate period. Thereafter, calves remained in TN conditions or were moved to HS conditions (THI: 75 to 83) for 19 d. Clinical assessments and body weight were recorded, and blood was routinely sampled. Organs from HS-Con and TN-Con calves were harvested at trial completion. Statistical analyses were carried out using the mixed model procedure of SAS (v9.4, SAS Institute Inc., Cary, NC). The statistical model included the fixed effects of body weight at birth, treatment, time, and their interactions as well as the random effect of calf.

Housing post-weaned Holstein calves in moderate heat stress conditions for 19 d markedly increased rectal (39.9, 39.9, 40.0 vs. 38.9 and 38.7°C; $P < 0.01$) and skin (38.7, 38.7, 38.8 vs. 32.8 and 31.8°C; $P < 0.01$) temperatures, as well as respiration rates (104, 104, 101 vs. 64 and 58; $P = 0.05$) of calves grouped in heat stress conditions (HS-Con, HS-Low, and HS-High, respectively) compared to calves housed in thermoneutrality (TN-Con and TN-PF, respectively). Exposure to high ambient temperatures significantly decreased dry matter intake of heat-stressed calves ($P < 0.01$). Calves in the HS-Con group consumed approximately 18% less feed than calves that were assigned to the TN-

Con group (P = 0.02). In accordance with our experimental design, TN-PF had similar dry matter intake as compared to HS-Con (P = 0.99). Although dry matter intake was comparable for the heat stress and pair-fed groups (HS-Con, HS-High and TN-PF), a low level of OA/PB supplementation presented an intermediate response and was similar to the observed intake of TN-Con (P = 0.20). Body weight was not modified by treatment; however, HS-Con and HS-Low had approximately 35% lower average daily gain, relative to TN-Con (P < 0.01). However, it is important to highlight that a high level of OA/PB supplementation (HS-High) during heat stress conditioning caused an intermediate response in average daily gain, which was similar to TN-Con (P = 0.16). Heat-stressed calves had lower small intestine (2.74 vs. 3.05 kg; $P \leq 0.15$) and liver weights (2.74 vs. 3.11 kg; $P < 0.05$), and greater kidney weights (686 vs. 589 g; $P < 0.10$) when compared to calves maintained in thermoneutrality. We conclude that reductions in dry matter intake account for losses in growth during heat stress and dietary OA/PB supplementation enhances heat stress resilience in calves.

Summary

Dietary organic acid and plant botanical supplementation is common practice in swine and poultry production, and science now suggests that we consider the practice in young dairy cattle. The justification is the consistent ability of OA/PB feeding to enhance growth, intestinal functionality, and reduce gastrointestinal bacterial pathogens. This said, microencapsulation of OA/PB to avoid rumen degradation of these compounds is likely needed in dairy cattle to elicit benefits in the lower gut. Our findings in Holstein calves are early evidence that dietary microencapsulated OA/PB feeding is a means to partially restore feed intake and average daily gain post-weaning when challenged by heat exposure. On-going investigations are examining whether dietary OA/PB influences the gastrointestinal bacteria profile in relation to changes growth performance.

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Exploratory Analysis of Haylage Quality Variability at Harvest

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Introduction

After corn silage, haylage is the most predominant ingredient in USA dairy diets (Kellogg et al., 2001). There are many factors during production, ensiling, and feedout that contribute to variation in haylage quality and the high inclusion rate means haylage is an important source of nutrient variability in TMR. To quantify haylage nutrient variation, we collected samples from alfalfa-grass mixtures on 7 New York dairy farms during the 2020 harvest. We used a mixed model to estimate the effect of farm, field, cut number, and weather on DM, CP, and NDF variability of haylage at harvest. The objective of our study is to identify the production factors that influence the variability in haylage quality at harvest. In the second phase of the trial, we will connect the variability at harvest to variability at feedout, in theTMR, and in milk production.

Main Findings

Haylage yield in summer 2020 was 34% lower than summer 2019 (NASS.USDA, 2019) due to low precipitation. The DM, CP, and NDF content of haylage at harvest were consistent with values reported by DairyOne (2019) across the three cuts. The mixed model analysis identified the farm (±5.7) and fields within farm (±4.1) as the largest random effects on DM % at harvest. The fixed effects showed DM decreased with increasing grass content (-0.14 ± 0.03), precipitation at harvest (-0.61 ± 0.24), and average solar radiation (-0.03 ± 0.01). Also, fixed effects showed DM increases with increasing average temperature at harvest (0.55 ± 0.19). Cut number was the largest source of random variation (±1.6) on CP%, followed by fields within farm (±1.0). CP content decreased with increasing grass content (-0.04 ± 0.00) and average solar radiation (-0.006 ± 0.002). Field was the largest random source of variation (±1.9) on NDF content, followed closely by farm (±1.3). The analysis of fixed effects shows significant effect of grass content (0.19 ± 0.01), dry time at the field (-1.63 ± 0.28), precipitation at harvest (-0.13 ± 0.06), and solar radiation (0.03 ± 0.00) on NDF.

Take Home Message

There is significant variation in DM, CP, and NDF content of haylage found between farms, between fields on the same farm, and between cuts. Quantifying the extent of this variation can help inform forage management and diet formulation decisions to improve nutrient delivery for precision feeding.

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Association Between Haptoglobin and Cow and Herd Level Outcomes

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Haptoglobin (HP) is an inflammation marker found in blood and is present at nearly zero concentration in healthy cows but increases over 100-fold at the onset of inflammation (Eckersall, 2000). Measuring HP in fresh cows may help us to identify cows that are at a greater risk of developing disease or provide additional tools for herd-level monitoring, which may help dairy producers improve their health management programs. Previous studies have found an association between elevated postpartum HP and disease, decreased milk production, and decreased reproductive performance; however, data are limited from large epidemiological studies (Huzzey et al., 2009; Dubuc et al., 2010; Huzzey et al., 2015; Nightingale et al., 2015). Therefore, the objectives were to 1) establish cow-level thresholds for HP concentrations to predict health disorders, 2) evaluate the association between elevated HP on milk production and reproductive performance, and 3) identify HP herd-alarm levels associated with herd-level changes in disease incidence, milk production, and reproductive performance.

Plasma samples were collected from 988 cows, 0 to 12 DIM, across 72 herds, and were analyzed for HP (University of Guelph Animal Health Laboratory). Results were previously reported by Kerwin et al. (2019; 2020). Cows with HP ≥1.52 g/L were 6.6 times more likely to be diagnosed with metritis (P = 0.001), HP ≥0.68 g/L were 4.9 times more likely to be culled within 30 DIM (P < 0.001), and HP ≥0.55 g/L were 2.5 times more likely to be diagnosed with metritis, clinical ketosis, a displaced abomasum, or any of the three disorders (P = 0.003). Cows with HP ≥0.55 g/L produced 386 kg less 305-d mature equivalent milk at the fourth test day (ME305; P = 0.004) and had a 25% decreased risk of conception by 150 DIM (Hazard ratio = 0.75; P = 0.002) than cows with low HP. Cows with elevated HP (≥0.55 g/L) had a median days to conception of 114 d compared to 101 d for cows with low HP (<0.55 g/L). Similarly, cows with HP ≥0.68 g/L were 0.80 times as likely to conceive at first service (P = 0.03). The herd-alarm level associated with disease incidence was defined as ≥20% of cows with HP ≥0.55 g/L, resulting in a 5.8 percentage unit increase in disease incidence (P = 0.01). The herd-alarm level associated with 21-d pregnancy rate was defined as ≥10% of cows with HP≥1.52 g/L, resulting in a 2.5 percentage unit decrease in 21-d pregnancy rate (P = 0.09). The herd-alarm level associated with conception risk at first service was defined as ≥20% of cows with HP ≥1.52 g/L, resulting in a 6.4 percentage unit decrease in conception risk at first service (P = 0.09). There was not a herd-alarm level associated with ME305 or 21-d conception risk.

Our results support previous research at the cow level and provides the opportunity to evaluate HP status at the herd level. Although previous work has established herd alarm levels for metabolites associated with energy balance, measuring HP at the herd level can be used as a herd health-monitoring tool and provides a unique opportunity to

address nutritional and non-nutritional challenges that may lead to increased inflammation.

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Valuating Nutritive Value of Alfalfa with Meadow Fescue Varieties for Optimal Quality in Dairy Production Systems

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Introduction

Dairy forage production systems in New York State are unique in that over 85% of alfalfa sown in the state is done in combination with a perineal grass. The soils in the state tend to have suboptimal drainage characteristics needed for optimal alfalfa production. Introducing a grass species into the alfalfa stand increases the neutral detergent fiber (NDFD) of the forage, and alfalfa-grass mixtures often have greater yield than pure alfalfa stands. Meadow fescue (*Festuca pratensis*), the underdog of perennial grasses in the US has recently been brought to the attention of forage extension specialists in the MidWest at the U.S. Dairy Forage Research Center, Dr. Michael Casler and at Cornell University, Dr. Jerry Cherney in the School of Integrated Plant Sciences, and Dr. Debbie Cherney in the Department of Animal Science. Originating from Europe, meadow fescue varieties show potential as a high-yielding, winter-hardy, high quality grass to be adopted into the dairy forage systems. The goals of the research were to 1) evaluate the growth of nineteen different meadow fescue varieties in combination with alfalfa while maintaining a 20-30% grass inclusion in the mix throughout the season, 2) achieve the highest possible quality of grass at harvest, and 3) provide region-specific management protocols to encourage home-grown forage use on dairy farms in the northeast.

2020 Growing Season and Take Home Message

Spring grass growth was delayed in 2020 due to drought conditions early in the season where grass development was delayed about 10 days. Subsequently rapid changes in plant development were observed as the stands phased into the reproductive mode (stem elongation with inflorescence) at a high rate. Average nutritive value of MF varieties in spring changed linearly between May 22 and May 30, 2020, where NDFD significantly decreased from 890 to 750 g/kg NDF (R^2 = 0.999) over the 8-day period at 1.7% units/day. Crude protein content in meadow fescue varieties declined at 0.7% units/day (R^2 = 0.99) and NDF concentration increased from 430 to 550 g/kg (R^2 = 0.996) over the 8-day period at a rate of 1.25% units/day. Grass proportion of alfalfa-grass mixtures ranged from 0.08 to 0.38 in the spring of 2019, and the range increased from 0.37 to 0.55 in the spring of 2020. The range in NDFD in the spring of 2020 was 7 g/kg, with Hidden Valley and Driftless ranking the highest. Research consistently highlights the potential economic advantage of improved grass to alfalfa forage quality where a 1% unit rise in NDFD translates to a 0.5 to 1 lb milk/cow/day increase in milk production. At this rate, everything needs to be done to ensure top quality forage is grown and harvested on time to achieve optimal forage yields and quality.

Impact of Starch and Energy on Amino Acid Requirements of Lactating Cows

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Introduction

The recognition of numerous metabolic interactions between energy and amino acid (AA) supply has become relevant regarding the efficiency of milk protein production (Lobley, 2007). Although these interactions at the post-absorptive levels can be complex, understanding the net supply of these nutrients relative the cows requirement can improve precision feeding, which in turn improves animal performance while reducing excessive nitrogen and AA supply fed (Lapierre et al., 2006). Improvements in the overall nitrogen (N) and AA efficiency of use is a metric widely used in the assessment of milk and milk component production given its implications in reducing both N inputs to cattle and a reduction in nutrient excretion into the environment. Previous calculations indicate that the efficiency of use for N averages around 25% of feed N intake and is highly variable, with ranges between 10% and 40% (Huhtanen and Hristov, 2009, Calsamiglia et al., 2010). Dijkstra et al. (2013) suggest that the upper limit for N efficiency of use for lactating cattle is above 40% and can be achieved through accurate predictions of AA requirements as well as a proper understanding of their biochemical interactions with energy and energy signaling mechanisms.

The disaggregation of metabolizable protein (MP) into the supply of each AA has become a precision feeding method to improve AA efficiency of use. Currently, the CNCPS v.7 predicts the requirements of each essential amino acid (EAA) and calculates the net supply of these EAA to determine animal productivity and assess first limiting nutrients. Further, the model expresses the requirements of each EAA relative to metabolizable energy (ME) to account for the energy demands for milk yield (Higgs and Van Amburgh, 2016). Application of this approach allows for a reduction in the MP supply, inherently dropping dietary crude protein (CP) and improving nitrogen use efficiency and allowing for the calculation of N requirements in two compartments, the rumen N requirement and the post-absorptive EAA requirement. Previous studies have formulated diets that are targeted for an optimum supply of EAA relative to ME and are lower in CP (13.5-14.5% DM) without compromising milk volume or protein yield relative to diets that have higher supply of EAA relative to ME and are consequently elevated in CP (Higgs et al., 2014, LaPierre et al., 2019). As a result, the nitrogen efficiency of use has improved for these diets which are targeted for the optimum supply of EAA; however, this improvement in efficiency is still not at the upper level described by Dijkstra et al. (2013).

To improve N and EAA efficiency of use in dairy cattle, it is important evaluate the level of glucogenic nutrients available to the animal. Literature has shown that increasing levels of glucogenic nutrients, including ruminally produced propionate and intestinally available glucose, has improved the post-absorptive transfer efficiency of AA from the

gastrointestinal tract to the mammary gland and allowed for greater yields of milk and milk protein (Lemosquet et al., 2010, Rius et al., 2010, Cantalapiedra-Hijar et al., 2016). Further, glucogenic nutrients might support N retention in peripheral tissue in the presence of elevated AA supply, improving the efficiency of productive use for N beyond the amount needed for milk protein production (Nichols et al., 2016, Curtis, 2018). Given the current understanding of glucogenic nutrients on AA utilization in dairy cattle, our objective was to evaluate the efficiency of use for EAA and N when supplying two levels of glucogenic nutrients in the form of ruminally produced propionate via differences in dietary starch, in addition to two levels of EAA supply. The EAA were formulated relative to ME (g digestible AA/Mcal ME) to assess if the optimum ratio of each EAA supply relative to ME changed with the addition of glucogenic nutrients. Our hypothesis was that cattle fed the higher level of glucogenic nutrients without the larger supply of EAA would have increased N efficiency through improved milk protein yield over cows with lower levels of glucogenic nutrients.

Methodology

To test the effect of dietary starch and EAA supply on lactation performance and N use efficiency, an experiment was conducted at the Cornell University Ruminant Center (Harford, NY) form December 2019-April 2020. The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals. One hundred and ninety-two Holstein cows (2.68 ± 1.37 lactations; 85 ± 26 days in milk; 672.2 ± 82.5 kg BW) were blocked in pens of 16 (n=12) by parity, days in milk, body weight, and previous lactation performance as part of randomized block design. Two enrollment periods, 96 cattle in Enrollment 1 [December 2019 – February 2020] and 96 cattle in Enrollment 2 [February 2020 – April 2020], were necessary to maintain the relevant period of lactation for observation. Each pen was fed TMR once daily at approximately 0630 h where pens were fed in the same sequence and targeted for a 5% refusal rate. All cattle were fed a common diet for a one-week acclimation period followed by a one-week covariate period in which baseline samples were taken to be used in the statistical analysis. Immediately following the covariate period, pens were randomly assigned one of four dietary treatments and fed for 7 weeks as part of the treatment period.

Dietary treatments included a 2 x 2 factorial design with two levels of dietary starch (23% [**LS]**and 29% [**HS**] DM) and two levels of essential amino acid supply (100% **[100]** and 105% **[105]** of the optimum grams of EAA per Mcal/ME requirement according to Higgs and Van Amburgh (2016). Diets were formulated using CNCPS v7 which predicts EAA requirements similar to Doepel et al. (2004) and Lapierre et al. (2007) but expresses requirements relative to ME (Higgs and Van Amburgh, 2016). Given the emphasis towards the evaluation of N and EAA efficiency of use, all diets were formulated to be isocaloric; however, diets did vary in the ingredients that supply energy and EAA. High starch (HS 100 and HS 105) diets were formulated with higher levels of starch containing ingredients, with a majority being a highly digestible steam flake corn, allowing for an increased pool size of fermentable starch in the rumen. To match the caloric density of the HS diets, the low starch diets (LS 100 and LS 105) were supplemented with a high palmitic form of Energy Booster (MSC Company, Dundee, IL), which did increase the

level of fatty acids consumed by those cattle (Table 1). Rumen unsaturated fatty acid load (RUFAL) was formulated to be similar in all four diets. Protein feeds were evaluated for intestinal digestibility using the Ross et al. (2013) assay to predict intestinally digestible N for more accurate predictions of EAA supply. Further, updated EAA profiles for commonly fed feeds determined within our lab (Van Amburgh et al., 2017) were implemented within the model to improve EAA supply predictions.

Table 1. Formulated EAA supply relative to megacalories of metabolizable energy

	Grams EAA:Mcal ME				
Essential Amino Acid	Higgs (2016)[1]	LS 100[2]	LS 105	HS 100	HS 105
Arginine	2.04	2.79	2.94	2.72	2.84
Histidine	0.91	1.12	1.16	1.10	1.19
Isoleucine	2.16	2.15	2.25	2.11	2.16
Leucine	3.42	3.18	3.37	3.20	3.32
Lysine	3.03	2.95	3.09	2.95	3.09
Methionine	1.14	1.11	1.18	1.11	1.18
Phenylalanine	2.15	2.09	2.21	2.06	2.12
Threonine	2.14	2.01	2.08	1.99	2.07
Tryptophan	0.59	0.60	0.62	0.59	0.61
Valine	2.48	2.34	2.43	2.30	2.39

[1] Optimum supply of EAA per Mcal ME according to Higgs et al. (2014)
[2]LS 100= Low starch, 100% EAA requirements; LS 100= Low starch, 105% EAA requirements; HS 100= High starch, 100% EAA requirements; HS 105= High starch, 105% requirements

Body weight and body condition score (1-5 scale) were measured and recorded weekly for all cattle. Milk samples were collected weekly during three consecutive milkings and analyzed for fat, true protein, lactose, total solids, and MUN (Dairy One, Ithaca, NY). A subset of cattle had milk samples taken at each milking to be analyzed for fatty acids (Barbano et al., 2014, Woolpert et al., 2016). Dry matter intake was determined daily for each pen as the difference between feed offered and refused (FeedWatch; Valley Ag Software). Samples of forages, TMR and refusals were sampled three times each week, composited, and analyzed for nutrient composition using near infrared reflectance spectroscopy. Additionally, feed ingredients included in the grain mixes were collected whenever new batches were delivered to the farm and analyzed by wet chemistry for chemical composition. A sub-sample of eight cows per pen were chosen for fecal spot sampling twice throughout the experiment. Eight samplings over a 3-day period (Day 1: 1300 h, 1900 h, Day 2: 0100 h, 0700 h, 1600 h, 2200 h, Day 3: 0400 h, 1000 h) were performed, compositing the eight cows into a single pen sample for each time point. Samples were processed and used to determine fecal N and estimate total tract NDF digestion using uNDF as an internal marker (Huhtanen et al., 1994, Raffrenato et al., 2018)

All statistical analysis was performed using SAS (v.9.4, SAS Institute Inc., Cary, NC). Feed and TMR chemistry results were produced via PROC TABULATE to provide mean, standard deviation, and standard error of all feed components and diets analyzed. Continuous measurements which were not repeated over time were subjected to ANOVA (PROC MIXED) with fixed effects including pen, level of starch, and level of protein.

Measurements taken over time were subjected to repeated measures ANOVA (PROC MIXED) using the same fixed effects with the added fixed effect of time. Cow within pen was considered random in both instances and any measurements taken within the covariate period of the experiment were utilized as a covariate measure within the models, where applicable. Values generated from CNCPS outputs are raw means.

Results and Discussion

Dietary Composition

Dietary ingredients and chemical composition of the four diets fed throughout the experiment are in Table 2. Observed dietary CP was slightly elevated over all four formulated dietary treatments, averaging 15.9% and 16.5% DM for 100% and 105% diets, respectively. Dietary starch observed for the LS diets were similar to the levels formulated for (23.4% formulated vs.23.7% observed) but observed starch levels for the HS diets were lower than formulation (29.1% formulated vs. 27.2% observed). We believe this discrepancy was caused by changes in starch content of the corn silage used throughout the first enrollment (29.5% DM), as this problem was corrected in the second enrollment period with corn silage of higher starch content (33.5% DM). Both LS diets had increased dietary fat over their HS counterparts, (4.5% LS vs 3.6% HS), allowing similar levels of ME intake (~68 Mcals ME/day). The LS diets also had increased levels of palmitic and stearic acid compared to the HS diets, corroborating with the supplementation of the high palmitic Energy Booster.

Daily supply of EAA and MP, as predicted by CNCPS v7 are in Table 3. The supply of most EAA increased from the 100% to 105% EAA requirement diets with the MP supply the 105% diets supplied at nearly five percent over the 100% diets. When evaluated against the optimums as defined by Higgs and Van Amburgh (2016), isoleucine, phenylalanine, tryptophan, and valine were not supplied at a level to maintain a 5% increase over the 100% EAA treatment and averaged about 4 units above the 100% treatment for those EAA. This demonstrates the learning that needs to occur to be able to formulate for each EAA at this precise a level as there are no rumen protected products on the market to simplify the formulation process.

In response to previous work, the supply of histidine was formulated to match or exceed the supply of methionine in these diets, which has been shown to improve lactation performance (Lee et al., 2012, Lapierre et al., 2014). It is also worth noting that although there was separation in the supply of arginine in the 100% and 105% diets, the grams relative to ME were significantly increased over the targeted optimum for this experiment (2.04 grams per Mcal ME). An *in vitro* study on casein and mTOR pathway related regulatory genes has suggested improved expression in the presence of elevated arginine (Wu et al., 2009, Wang et al., 2014), suggesting non-nutritive functionality of this AA which might improve milk protein yield in this experiment. The deviations from the targeted supply of EAA highlight the difficulty in balancing for all EAA in lactating diets,

particularly given the constraints on farm feed inventories, feed ingredient amino acid profiles, and the variability of feed chemistry for the available feeds.

Table 2. Ingredients and chemical composition of experimental diets

Ingredient, % DM	LS 100[1]	LS 105	HS 100	HS 105
Corn silage	52.61	50.09	42.37	40.03
Mixed grass/Legume silage	8.01	9.94	9.54	7.24
Steam flaked corn	4.19	4.40	12.41	12.20
Corn meal	2.10	3.14	5.34	7.89
Beet pulp	6.86	4.55	1.91	---
Wheat midds	4.29	4.32	7.25	3.81
Canola	3.62	1.15	1.91	7.62
Soybean meal	7.24	9.56	10.88	6.86
SoyPLUS[2]	5.53	7.27	0.95	3.05
Soybean hulls	0.67	1.34	3.63	7.43
Energy Booster HP	1.33	0.96	---	---
Dextrose	0.19	---	0.38	0.38
Urea	0.23	0.19	0.19	0.17
Smartamine M[3]	0.09	0.10	0.08	0.08
Smartamine ML[4]	---	0.04	0.04	0.08
Minerals and Vitamins	3.04	2.96	3.12	3.15
Observed Chemical Composition[6], % DM ± standard deviation				
DM	36.4 ± 1.1	36.5 ± 1.3	38.9 ± 1.7	42.4 ± 3.8
CP	15.9 ± 0.4	16.6 ± 0.6	15.9 ± 0.8	16.4 ± 0.5
NDICP, % CP	16.0 ± 0.2	15.1 ± 0.2	13.7 ± 0.3	14.3 ± 0.4
ADICP, % CP	6.1 ± 0.4	5.5 ± 0.4	5.4 ± 0.8	5.5 ± 0.3
Soluble protein, % CP	40.1 ± 1.5	39.5 ± 1.4	38.7 ± 3.1	37.3 ± 1.2
RUP, % CP	30.0 ± 0.7	30.3 ± 0.7	30.7 ± 1.5	31.4 ± 0.6
Sugar	4.3 ± 0.4	4.5 ± 0.4	4.5 ± 0.6	4.6 ± 0.4
Starch	23.9 ± 1.4	23.4 ± 1.6	27.0 ± 2.3	27.4 ± 2.6
Starch digestion 7hr, % Starch	76.7 ± 2.1	75.2 ± 2.1	77.4 ± 2.9	77.4 ± 1.4
NFC	42.4 ± 0.3	41.9 ± 0.4	44.3 ± 0.5	44.8 ± 0.4
aNDFom	32.2 ± 1.2	31.9 ± 1.6	31.3 ± 2.2	30.6 ± 1.6
uNDF240, % NDF	27.0 ± 1.4	24.9 ± 1.5	23.6 ± 3.5	25.2 ± 1.5
Ether Extract	4.5 ± 0.2	4.5 ± 0.1	3.6 ± 0.3	3.6 ± 0.2
TFA	3.1 ± 0.3	3.0 ± 0.1	2.4 ± 0.2	2.5 ± 0.2
C16:0, TFA	24.2 ± 2.4	22.0 ± 2.3	16.5 ± 3.4	17.3 ± 2.0
C18:0, TFA	5.5 ± 0.4	4.9 ± 0.5	3.5 ± 0.7	4.0 ± 0.7
Ash	7.7 ± 0.4	7.8 ± 0.5	7.6 ± 0.5	7.5 ± 0.5
ME, Mcal/kg	2.61	2.61	2.63	2.64
Pool Size Based on Intake				
Sugar, kg/day	1.12	1.17	1.26	1.31
Starch, kg/day	6.27	6.02	7.55	7.75
aNDFom, kg/day	8.44	8.24	8.74	8.65
Total Fatty Acids, g/day	964.6	894.2	641.4	669.5
RUFAL Load, g/day	507.6	517.9	491.9	523.0

[1]LS 100= Low starch, 100% EAA requirements; LS 100= Low starch, 105% EAA requirements; HS 100= High starch, 100% EAA requirements; HS 105= High starch, 105% requirements

[2] SoyPLUS (West Central Cooperative, Ralston, IA) rumen protected soybean meal

[3] Smartamine M (Adisseo USA Inc, Alpharetta, GA) rumen protected Met (100% AANt)

[4] Smartamine ML (Adisseo USA Inc, Alpharetta, GA) rumen protected Lys (75 % AAN) and Met (25% AAN)

[6] Chemical components are expressed as % DM unless stated. ADICP = CP insoluble in acid detergent; NDICP = CP insoluble in neutral detergent; RUP = Rumen undegraded protein (model predicted), NFC = non-fiber carbohydrates, aNDFom =- amylase and sodium sulfite treated NDF corrected for ash residue, uNDF240 = undigested NDF after 240 hours of in vitro fermentation, ADL = acid detergent lignin, EE = ether extract, TFA = total fatty acids.

Animal Performance and Efficiency

Differences were observed in dry matter intake (DMI) between the cows on the LS and HS diets (P = 0.01) as cattle receiving the HS diets consumed over 2 kg more DM relative to the cattle on LS diets. Differences in these DMI might be attributed to a larger proportion of forage dry matter and NDF fed to the cows on the LS diets (not shown). This could have attributed to increased levels of dietary aNDFom in the LS compared to HS diets (Table 2) and it is likely that these cattle reached a physical fill limitation over their HS counterparts (Cotanch et al., 2014). Also, depression of DMI has been observed in studies where fat was infused post-ruminally (Bremmer et al., 1998, Drackley et al., 2007), although the level of palm fatty acid infusion in these studies was far above the dietary supplemented levels observed in the current study. Additionally, to maintain appropriate intake levels without elevating the caloric density, the cows fed the HS diets were fed more fibrous non-forage ingredients, including soyhulls. Although the rate of degradation of soyhulls is reasonably slow (~0.05/h), their extent of digestion is high (~90% of aNDFom) and added to the increased DMI as they contributed less to an aNDFom physical fill limitation. Rumination time of cattle supports this as the cows fed the LS diets tended to ruminate more with an average of 30 more minutes per day (650 vs. 622 minutes per day; P = 0.09). Cattle consuming a higher supply of EAA had significantly high milk volume and energy corrected milk (P = 0.01), with the HS 105 diet yielding greater volume and components compared to other treatments and this follows both the higher starch and greater intake.

Review of the component yields suggest that the higher ECM production for both 105% diets was achieved via yields of different components in the milk. To start, milk true protein yield was increased for cows consuming the HS diets (1.45 kg vs. 1.36 kg; P = 0.01), which is in support of our hypothesis. Milk protein output was highest in cattle fed the HS 105 diet, which is in support of previous findings where the supplementation of AA and glucose precursors have stimulated milk protein output (Raggio et al., 2006). Conversely, cows fed the LS diets had significantly greater yields of milk fat throughout the experiment (1.85 kg vs. 1.78 kg; P = 0.01). The improvements in milk fat secretion is undoubtedly due to the supplementation of fat in the diet, contributing to a greater level of lipogenic nutrients in the diet. Milk samples sent for fatty acid analysis suggest that there was a greater proportion of preformed fatty acids in the milk of cattle fed the LS diet (31.2% vs 29.5%; P = 0.01) whereas cattle on the HS diet produced a greater proportion of de novo fatty acids (26.8% vs. 25.2%; P = 0.01). Dietary fat supplementation has shown to influence the milk fat composition by improving the level of preformed fatty acids available for milk fat yield (Stoffel et al., 2015). Milk urea nitrogen (MUN) levels were lowest in LS 100 cows and highest in LS 105 cows. This difference in MUN is likely due to the corn silage starch levels in the first period, which supplied lower levels of rumen fermentable starch, possibly reducing microbial activity compared to what was initially formulated, however other interactions will be explored once the feed chemistry is evaluated via the CNCPS v.7 evaluations.

The initial BW of cows was not different for all treatments (P = 0.90; Table 4); however, cow fed the LS diets tended to have a higher final BW compared to cows on the HS diets (698.4 kg vs. 693.4 kg; P = 0.09). Body condition scores of HS cows were significantly lower than LS cows at the beginning of the experiment but were not different at the final measurement for the experiment. Feed efficiency (Milk yield:DMI) and ECM feed efficiency were significantly higher for cows fed the LS diets, with the HS 105 diet having the lowest feed efficiency. The lower level of feed efficiency can again be attributed to higher levels of DMI as cattle on this diet were those who had the best lactation performance. Alternatively, it has been well documented that an increase in feed efficiency is observed when supplemental fat, in the form of palmitic acid, is fed (Rico et al., 2014, Boerman et al., 2015, Nichols et al., 2018a). This might have influenced our results separate from the efficiency of use for N. Efficiency of use for feed N into milk N was higher for cows fed at 100% EAA requirements, with HS 100 having the highest N efficiency (32.5%; P = 0.04). The current literature has shown that an increase in AA supply improves milk protein output, but at the cost of N use efficiency (Dijkstra et al., 2013, Apelo et al., 2014). This is in support of our current hypothesis as it suggests that the optimum grams of EAA per Mcal of ME is creating better efficiency of use for these EAA over a higher supply of EAA fed in the 105% diets and that supplying more EAA does not always result in greater milk protein synthesis.

Table 3. Daily supply of essential amino acids for each treatment diet as calculate using CNCPS v7 using actual feed chemistry and dry matter intakes.

Essential Amino Acid, grams	LS 100[1]	LS 105	HS 100	HS 105
Arginine	190.9	201.3	186.2	194.5
Histidine	76.6	79.0	75.0	81.2
Isoleucine	147.0	154.2	144.3	147.7
Leucine	217.8	230.3	218.7	226.8
Lysine	202.0	211.5	201.5	211.6
Methionine	76.1	80.6	75.9	80.5
Phenylalanine	142.9	151.1	140.7	145.3
Threonine	137.9	142.3	136.4	141.8
Tryptophan	40.9	42.4	40.7	41.9
Valine	160.2	166.5	157.4	163.7
Total EAA	1392.3	1459.2	1376.8	1435.0
MP Supply	2872	3005	2852	2980

[1]LS 100= Low starch, 100% EAA requirements; LS 100= Low starch, 105% EAA requirements; HS 100= High starch, 100% EAA requirements; HS 105= High starch, 105% requirements

Conclusions

It is apparent that the production of milk protein increased as cows were fed the HS diets, supported by an increased supply of EAA; however, those cows also consumed significantly more feed, which would provide for both more glucogenic substrates and greater microbial yield, which would supply even greater EAA. This improvement in milk protein output by the increase in EAA supply in the HS 105 diets occurred while decreasing the efficiency of N utilization compared to the other diets (Table 4). In contrast, cows fed the HS 100 diet had the highest level of N efficiency compared to other treatments and a reasonable but slightly lower milk protein output by approximately 50

g/d. This data supports the hypothesis that greater glucogenic substrates support greater milk protein synthesis and further indicate the optimum EAA values per unit of ME are reasonable but there are some EAA that are required at higher levels to support the energy signaling for greater protein synthesis. Nichols et al. (2018b) recently presented similar findings where the postruminal supplementation of glucogenic precursors improved milk N efficiency at both a low level of MP supply (75% of requirements) and higher level of MP supply (120% of requirements). Given that we were not able to fully meet the balanced requirements for all the EAA at the 105% level, these small deficiencies might explain why the milk protein response was not greater than observed and that milk N efficiency was decreased. Further work to evaluate this interaction between glucogenic supply and milk protein synthesis will have to ensure that all EAA requirements are effectively met. However, this data does suggest the optimum requirements as described by Higgs and Van Amburgh (2016) is a good starting point in formulation of EAA supply relative to ME for lactating dairy cattle.

Improvements in milk fat output and feed efficiency for cattle fed the low starch diets should not be disregarded in light of the improved efficiency of use for N in the HS 100 diet. A body of literature exists that describes similar improvements in feed efficiency when diets are supplemented with lipogenic nutrients and more work is needed to evaluate the effect of fat and fatty acid supplementation when diets are balanced for EAA. Further work, including analysis of plasma samples for urea nitrogen, AA, and insulin content, is also needed to provide data to describe the metabolic signaling and metabolites related to the diets in the current study. Findings from this work will be used for CNCPS v7 model evaluation and allow for refinements in predicted EAA requirements of lactating dairy cattle.

Figure 1. Effect of dietary treatment on milk, energy corrected milk, and component yield for animals fed.

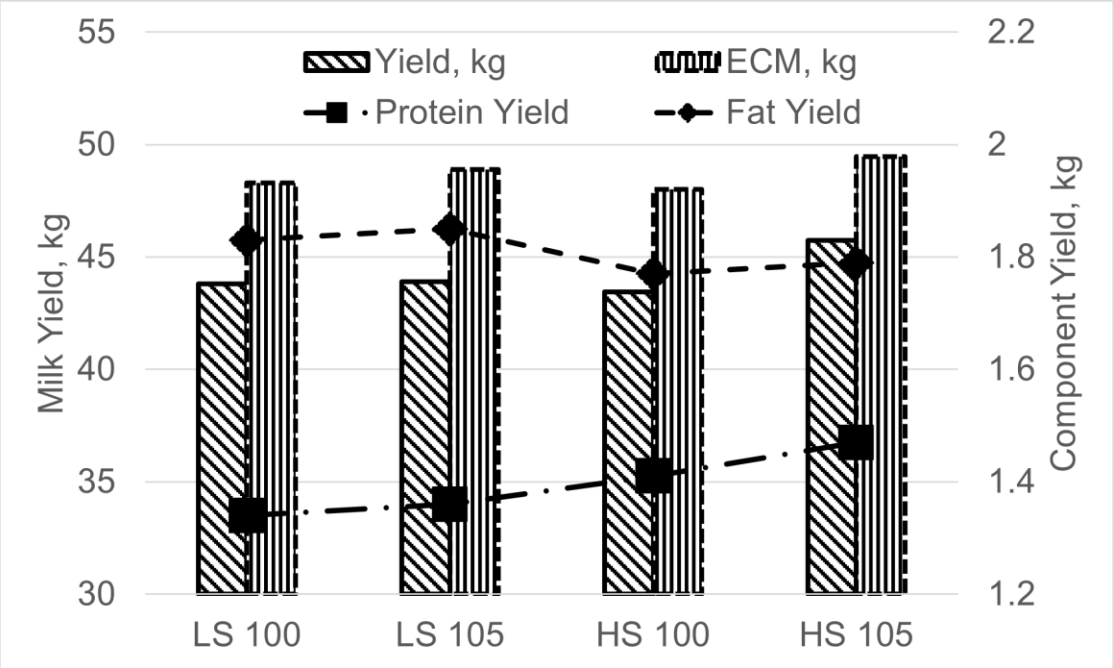

Table 4. Effects of treatment diets on milk production, intake, body measurements, and efficiencies

	Dietary Treatments					P-Values				
	LS 100[1]	LS 105	HS 100	HS 105	SEM	Starch	AA	Starch*AA	Time	Starch*AA*Time
Intake and milk production, kg/d										
Dry matter intake	26.65	26.35^{y}	28.95^{x}	28.34	0.66	0.01	0.54	0.83	0.73	0.54
Energy correct milk yield[2]	48.31^{a}	48.90^{ab}	48.02^{a}	49.48^{b}	0.33	0.66	0.01	0.20	0.01	0.65
Milk yield	43.81^{a}	43.92^{a}	43.46^{a}	45.73^{b}	0.26	0.01	0.01	0.01	0.01	0.06
True protein yield	1.35^{a}	1.37^{a}	1.42^{b}	1.47^{c}	0.01	0.01	0.01	0.09	0.01	0.25
Fat yield	1.83^{ab}	1.86^{b}	1.77^{a}	1.79^{ab}	0.02	0.01	0.32	0.76	0.01	0.21
Lactose yield	2.16^{a}	2.17^{a}	2.14^{a}	2.26^{b}	0.01	0.01	0.01	0.01	0.01	0.85
Milk composition, %										
True protein	3.08^{a}	3.13^{a}	3.27^{b}	3.25^{b}	0.02	0.01	0.56	0.02	0.01	0.01
Fat	4.20^{a}	4.26^{a}	4.09^{b}	4.00^{c}	0.04	0.01	0.72	0.07	0.19	0.04
Lactose	4.94	4.93	4.93	4.94	0.007	0.59	0.68	0.79	0.01	0.85
MUN, mg/dL	10.3^{a}	13.9^{b}	11.9^{c}	11.2^{d}	0.15	0.01	0.01	0.01	0.01	0.02
Fatty acid composition, %										
De Novo	25.0	25.3	26.9	26.7	0.27	0.01	0.90	0.38	0.01	0.01
Mixed	43.1	42.6	43.9	42.8	0.37	0.13	0.04	0.42	0.01	0.01
Preformed	31.5^{a}	30.9^{ab}	29.0^{c}	30.0^{bc}	0.38	0.01	0.54	0.03	0.01	0.76
Body Measurements										
Initial Body Weight, kg	676.0	678.9	680.2	686.1	12.5	0.65	0.72	0.90	---	---
Final Body weight, kg	698.5	698.2	696.1	690.7	2.95	0.09	0.34	0.39	---	---
Initial BCS, 1-5 Scale	2.93	2.89	2.83	2.88	0.02	0.02	0.90	0.11	---	---
Final BCS, 1-5 scale	3.00	2.96	3.01	3.00	0.04	0.56	0.50	0.63	---	---
Rumination Time, min/day	655.0	646.3	634.5	610.4	16.2	0.09	0.31	0.64	0.24	0.35
Efficiencies										
Feed Efficiency	1.65^{a}	1.66^{a}	1.59^{b}	1.54^{c}	0.02	0.01	0.03	0.01	0.01	0.01
ECM Feed Efficiency	1.82^{a}	1.85^{a}	1.75^{b}	1.67^{c}	0.02	0.01	0.05	0.01	0.01	0.01
Milk Nitrogen:Feed Nitrogen, %	32.2^{ab}	32.0^{a}	32.5^{b}	31.2^{c}	0.20	0.31	0.01	0.04	0.01	0.01

^{a,b,c} Denotes statistical significance ($P \leq 0.05$) ^{x,y,z} Denotes statistical tendencies ($P \leq 0.10$)

[1]LS 100= Low starch, 100% EAA requirements; LS 100= Low starch, 105% EAA requirements; HS 100= High starch, 100% EAA requirements; HS 105= High starch, 105% requirements [2]Estimated according to Tyrrell and Reid (1965)

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Trimethylamine *N*-oxide in Humans and Dairy Cows: Should We Be Concerned?

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Introduction

The Greenland shark is an oceanic enigma. The creature holds the recognition as being the longest-living vertebrate in the world. They sexually mature after 100 years and survive for over four centuries. The shark also contains some of the highest biological observed tissue concentrations of a metabolite called trimethylamine *N*-oxide (TMAO). Although poisonous when consumed fresh, Greenland shark is compressed and dried to lower the TMAO content and produce a fermented and odorous food called hákarl. These ancient 'shark bites' are unique but it is the TMAO that has garnered the recent attention of the scientific community. This is because TMAO has been labeled "The New Red-Meat Risk" for heart disease (Abbasi, 2019). Indeed, numerous studies have been published linking higher circulating TMAO concentrations with cardiovascular disease but also non-alcoholic fatty liver disease (NAFLD) in humans (Li et al., 2017b, Roncal et al., 2019, Tan et al., 2019); however, the science is contentious and subject to major criticism. Research has centered on the ability of dietary L-carnitine, choline, or betaine found in red meat, dairy products, chicken, eggs, and fish to be broken down to trimethylamine (TMA) in the gut, which is absorbed and converted to TMAO in liver by the enzyme flavin-containing monooxygenase-3 (FMO3). For the dairy industry, the story of TMAO has several implications. First, increases in endogenous TMAO may indirectly reflect the gastrointestinal degradation and limited bioavailability of choline, betaine, or L-carnitine, which are often fed as rumen-protected supplements to dairy cattle. Second, TMAO may elicit direct effects on bovine metabolism and thus influence milk production or health of the animal. Third, milk and dairy products are a potential source of TMAO and TMAO-precursors such as choline, and thus represent a potential concern for consumers questioning their own dairy intake. This review breaks down the current understanding of TMAO in humans and dairy cows. The associative and causative role of TMAO within the development of human disease is considered with an emphasis on potential mode of action. Studies focused on the relationship between dairy consumption and TMAO are considered with the realization that a single dietary component alone, like dairy, is not enough to influence disease progression.

Trimethylamine *N*-Oxide Metabolism and the Gut Microbiome

Dietary choline (free or lecithin-derived), betaine, and L-carnitine are converted to TMA within the small intestine by choline TMA lyase, betaine reductase, and carnitine oxidoreductase, respectively (Figure 1). Other TMA precursors include γ-butyrobetaine (from betaine degradation) and ergothioneine. Research has shown that bacterial TMA-forming enzymes are ubiquitously present in mammals; but their abundance is influenced

by the composition of the gut microbiota and diet (Rath et al., 2020). For instance, carnivores have a higher fecal abundance of carnitine oxidoreductase than herbivores (Rath et al., 2020). Once TMA is formed, the tertiary amine is absorbed through the intestinal epithelium via passive transport. This includes ingested TMAO that avoids direct absorption but is converted to TMA by TMAO reductase. Once in portal circulation, TMA enters the liver where it is oxidized to TMAO by NADPH-dependent FMO3. FMO3 is one of five functional FMO genes in humans and the isoform predominantly expressed in liver. Approximately 95% of all TMA that enters the liver is converted to TMAO. In a study of humans administered radiolabeled TMA or TMAO, 95% of TMAO was is excreted in urine in a 3:95 TMA:TMAO ratio (Al-Waiz et al., 1987). Minimal TMAO is excreted in feces and breath (4 and 1%, respectively; Al-Waiz et al., 1987).

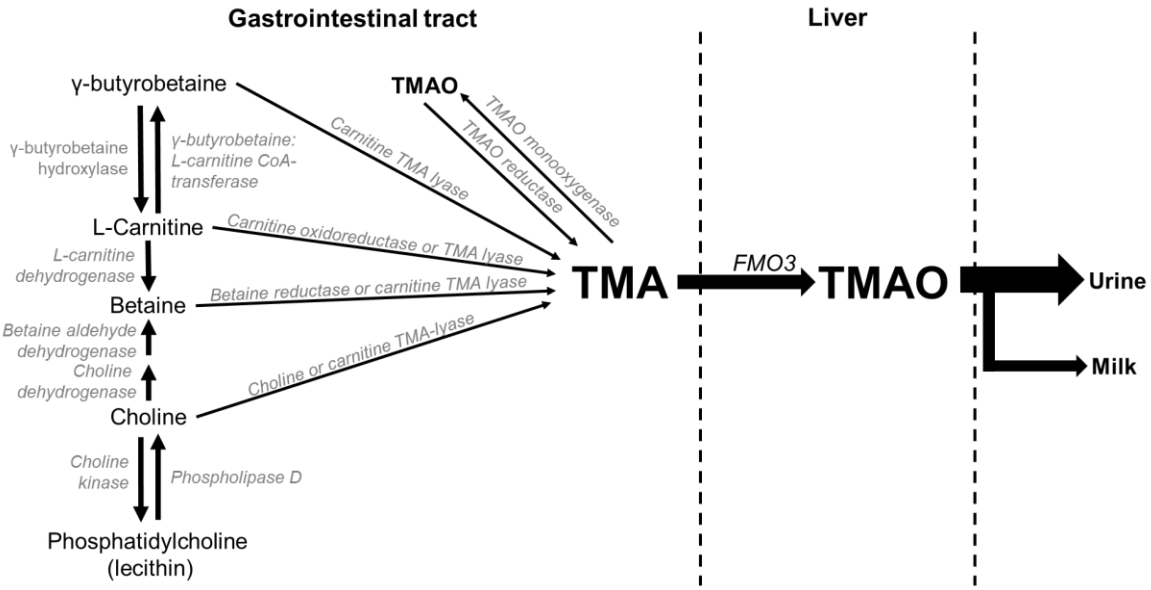

Figure 1. The synthesis of TMAO from choline, betaine, and L-carnitine in mammals. The gastrointestinal synthesis of dimethylamine from TMAO is not shown. Adapted from Janeiro et al. (2018) and modified.

The gut microbiome is the major influencer of endogenous TMAO status in mammals. For example, the bacterium *Desulfovibrio desulfuricans* is recognized for expressing choline TMA lyase (Craciun and Balskus, 2012). Other bacterial species that reside in the human gastrointestinal tract that generate TMA include *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda* (Romano et al., 2015). Mice fed L-carnitine displayed increases in TMA and TMAO production in association with an increased population of *Bacteroides*, *Parasutterella, Staphylococcus,* and *Ruminococcaceae* within the intestinal tract (Koeth et al., 2014). When comparing the effects of dietary choline versus L-carnitine on the cecal microbiota at the phylum level, the abundance of *Desulfovibrio* was greater in choline-fed mice, whereas the abundance of *Acinetobacter* was greater in L-carnitine-fed mice (Yu et al., 2020). Diet plays a role in modulating the gastrointestinal microbiome and

supplies choline, betaine, and L-carnitine, which together influence TMA formation (described further below). In addition to diet, the use of broad spectrum antibiotics is also likely to influence TMA and TMAO production via direct effects on the gut microbiome (Tang et al., 2013). However, it is conceivable that any intervention that influences the gastrointestinal microbiome would potential influence TMA and TMAO formation such as heat stress, exercise, pre- and probiotic therapy, pharmaceutical use, or malnutrition.

Trimethylamine *N*-Oxide, Human Health, and Diet

In humans, TMAO has been implicated in the progression of cardiovascular disease and NAFLD in humans (Ufnal et al., 2015, Li et al., 2017b, Abbasi, 2019, Roncal et al., 2019). Currently, the involvement of TMAO in the development of cardiovascular disease has the most scientific support. It has been suggested that the heightened mortality risk and cardiovascular risk linked to the chronic consumption of red meat, which is high in L-carnitine, is attributed in part to the conversion of L-carnitine to TMAO (Abbasi, 2019). A meta-analysis of published prospective studies suggests individuals with high circulating TMAO concentrations have a 62% increased risk for acquiring a major cardiovascular adverse event (Heianza et al., 2017). The thought is that TMAO accelerates atherosclerosis by increasing arterial plaque formation. The mechanisms of cardiac impairment caused by TMAO may involve reduced cholesterol clearance in bile and increased cholesterol-laden foam cells (Warrier et al., 2015, Tomlinson and Wheeler, 2017). Furthermore, microbial transplantation with human gut commensals containing choline TMA-lyase was adequate to transmit increased platelet reactivity and thrombosis in germ-free mice (Skye et al., 2018). TMAO also causes vascular inflammation and disrupts redox homeostasis to cause oxidative stress (Li et al., 2017a). However, critiques are quick to question whether TMAO causes cardiovascular disease. In support of their argument, chronic, low-dose oral TMAO reduces diastolic dysfunction and heart fibrosis in hypertensive rats (Huc et al., 2018). In addition, elevations in circulating TMAO may be indicative of other cardiovascular disease risk factors including high salt intake (Bielinska et al., 2018), a low glomerular filtration rate (Missailidis et al., 2016), and a compromised gastrointestinal barrier (Kindie et al., 2017, Santisteban et al., 2017). Moreover, diets high in fish have been considered “heart healthy” but fish has high amounts of TMAO and omega-3 fatty acids, which is counterintuitive. The rebuttal is that TMAO is only elevated in deep-sea fish like cod and halibut, but not present in fresh-water fish like bass, catfish, and trout (Abbasi, 2019). Others also argue that TMAO predicts for the future development of heart disease, which can occur in patients with normal kidney function (Abbasi, 2019). At this point in time, we can conclude that TMAO does accumulate in patients with cardiovascular injury but we cannot conclude whether TMAO acts in a causative manner independent of other heart disease risk factors.

High circulating concentrations of TMAO have also been observed in patients with NAFLD (Chen et al., 2016, Tan et al., 2019), which is characterized by excessive hepatic triglyceride deposition and inflammation. In mice fed a high-fat diet, feeding TMAO increased hepatic triglyceride accumulation (Tan et al., 2019). One possibility is that TMAO may alter hepatic bile acid production to inhibit farnesoid X receptor signaling and promote lipogenesis to trigger NAFLD (Wilson et al., 2016, Tan et al., 2019). However,

the oral administration of TMAO has also been shown to impair glucose tolerance in mice fed a high fat diet (Gao et al., 2014) and diabetic individuals have high TMAO plasma concentrations (Dambrova et al., 2016); so, TMAO could enhance adipose tissue lipolysis and hepatic fatty acid uptake by inhibiting insulin action. Such findings are supported by elevated circulating TMAO concentrations in obese patients with non-alcoholic steatohepatitis (an inflammatory form of NAFLD) and type 2 diabetes (León-Mimila et al., 2020). But controversy is still present because choline and betaine, TMAO precursors, are recognized as key dietary nutrients for the prevention of NAFLD. Humans that eat low choline diets develop NAFLD (Corbin and Zeisel, 2012, Guerrerio et al., 2012). Dietary betaine has also been shown to improve or protect against NAFLD in humans (Abdelmalek et al., 2009, Kathirvel et al., 2010). Choline and betaine support the hepatic synthesis of phosphatidylcholine, which is a glycerophospholipid needed for the assembly and secretion of very-low-density lipoproteins containing triglyceride (McFadden et al., 2020). So, an increased TMAO status may once again reflect underlying conditions of the disease but TMAO may not act in a causative manner. In agreement, NAFLD has been linked to renal function impairment, which would prevent TMAO excretion (Le et al., 2019). Gut microbial dysbiosis and a disrupted intestinal barrier are also likely at play during NAFLD (Farhadi et al., 2008, Boursier et al., 2016, Soderborg and Friedman, 2019), and such outcomes would influence endogenous TMAO status (Ufnal and Pham, 2017, Xu et al., 2017, Wang et al., 2019).

Although the effects of TMAO on health is riddled with uncertainty, diet clearly influences TMAO status in humans. In a study of Swedish men, consumption of fish from the Baltic sea was associated with increased urinary TMAO concentrations (Svensson et al., 1994). Diets high in resistant starch increase plasma concentrations of TMAO as well (Bergeron et al., 2016). The Paleolithic diet is based on the consumption of meat, fish, eggs, and fruits and vegetables without processed foods, grains or dairy products. Long-term adherence to the Paleolithic diet was associated with different gut microbiota and increased serum TMAO concentrations (Genoni et al., 2020); although not observed by Genoni et al. (2019). The chronic consumption of red meat increased plasma and urine TMAO concentrations as compared to diets containing non-meat protein (Wang et al., 2018). Concentrations of plasma and urinary L-carnitine, but not choline, were also linked to red meat consumption and increased TMAO in this study. Oral L-carnitine supplementation has been shown to increase plasma TMAO status (Miller et al., 2016), and oral choline bitartrate supplementation has been show to increase fasting plasma TMAO concentrations in parallel with platelet aggregation (Zhu et al., 2017). The consumption of egg yolks (0 to 6 yolks containing 0 to 714 mg of total choline) has been shown to increase plasma and urine TMAO concentrations in humans enrolled in a longitudinal, double-blind, randomized dietary intervention study (Miller et al., 2014). They estimated that ~11 to 15% of dietary total choline was converted to TMAO. In a German adult population, meat, egg, or fish consumption was not associated with plasma TMAO status; however, increases in milk and dairy food consumption was related to increased plasma TMAO concentrations (Rohrmann et al., 2016). In addition, the consumption of fermented dairy products (e.g., yogurt) lowered the plasma and urinary TMAO postprandial response as compared to the intake of non-fermented milk (Burton et al., 2020). We can agree that diet modulates TMAO supply in humans; however, at this time,

it would be potentially damaging to suggest that a specific food impairs human health because of the TMAO response it elicits.

Trimethylamine *N*-Oxide, Choline Bioavailability, and Dairy Cow Health

Our understanding of the role of TMAO in dairy cows is in its infancy. The scientific field has more questions than answers; however, some early insights suggest the need to learn more. Classic work by Sharma and Erdman (1989) demonstrated that unprotected choline is extensively degraded in the rumen (>97%). Choline can be converted to methylamine and TMA in the rumen by microorganisms (Neill et al., 1978). TMA is further metabolized to methane (Neill et al., 1978); albeit, TMA may accumulate in rumen fluid during fasting. Data obtained by studying the human gut suggests that choline degradation is not evenly distributed between common phyla. For instance, choline utilization gene clusters (containing choline TMA-lyase) are found in *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, and absent from *Bacteroidetes* (Craciun and Balskus, 2012). Regardless, choline is degraded in the rumen to TMA. This said, a recent study by our lab at Cornell University demonstrated that the abomasal infusion of unprotected choline chloride increased plasma TMAO concentrations in lactating dairy cows (Myers et al., 2019). These findings suggest that post-ruminal degradation of choline to TMA likely occurs in a manner similar to non-ruminants. Moreover, TMA produced from choline is likely converted to TMAO in the bovine liver. In another study, the dietary supplementation of deoiled soy lecithin containing phosphatidylcholine was able to increase plasma TMAO concentrations in mid-lactation dairy cows fed fractionated palm fatty acids (Fontoura et al., 2020). These data highlight the possibility that complex lipids that contain a choline moiety may also increase TMA and TMAO production in the dairy cow. One concern is that choline degradation in the lower gut has the potential to limit choline bioavailability. Rumen-protected choline supplements were developed to ensure that choline avoided rumen degradation but choline released in the intestinal lumen could be degraded by intestinal bacteria and limit choline availability for absorption. Work by de Veth et al. (2016) estimated that the net absorption of choline when infused into the abomasum as unprotected choline chloride was 61%. Because choline transport by carrier-mediated transport (at low concentrations) or passive diffusion (at high concentration) is likely adequate to absorb choline at current feeding levels (Sheard and Zeisel, 1986, de Veth et al., 2016), the difference could be attributed to the microbial conversion of choline to TMA in the gut lumen. Based on the above described study in humans (Miller et al., 2014) and the work by de Veth et al. (2016), intestinal degradation of choline in the cow could range from ~10 to 40%, but this is purely speculative at this point in time.

The TMA that enters the cow is most likely converted to TMAO in the liver. We hypothesis that this occurred in our trials (Myers et al., 2019, Fontoura et al., 2020). In support, a nonsense mutation in the hepatic FMO3 gene is responsible for the accumulation of TMA in the Swedish Red and White dairy breed (Lundén et al., 2002). This defect elicits a fishy off-flavor in milk produced by these cows that smells like rotting fish. One limited finding was the observation that circulating TMAO concentrations are elevated in cows with fatty liver disease (Xu et al., 2016). This outcome is supported by

the observed accumulation of TMAO in non-ruminants with fatty liver (Chen et al., 2016, Tan et al., 2019, León-Mimila et al., 2020). High TMAO status in fresh cows could be a concern because fatty liver disease is a common postpartum health condition. Moreover, rumen-protected choline feeding was designed in part to alleviate fatty liver in dairy cows; but the hepatic triglyceride-lowering effect of choline is inconsistently observed (Arshad et al., 2019, McFadden et al., 2020), which may be due to differences in TMAO status. To begin our focused studies on TMAO, we sought to define the effects of TMAO in early lactation dairy cows on measures of metabolic health including liver enzymes, glucose tolerance, and milk production.

The effects of acute intravenous TMAO infusion in early lactation dairy cows

At the Cornell University Dairy Research Center (Harford, NY), eight early lactation Holstein dairy cows (30.4 ± 6.41 days in milk; 2.88 ± 0.83 parity) were enrolled in a 4 × 4 replicated Latin square design. Cows were intravenously infused TMAO solubilized in saline at four different concentrations: 0 (control), 20, 40, or 60 g/d for 6-d experimental periods with 9-day washout period to avoid carryover effects. Pre-prandial plasma and serum samples were collected daily. Milk samples were collected on day -1, 0, 5, and 6, relative to start of TMAO infusion. Urine was collected on day -1 and 6. An intravenous glucose tolerance test was administered and liver biopsies performed on day 5 and 6 of each experimental period, respectively. Plasma TMAO, triglyceride, total fatty acid, and glucose concentrations were quantified. A liver serum panel was also performed to assess liver health. Circulating metabolites and proteins were analyzed in SAS (Version 9.4; SAS Institute Inc., Cary, NC) utilizing the MIXED model procedure. The mixed model included the fixed effects of baseline measurements as a covariate and treatment. Milk production data were analyzed using a similar approach. Untargeted lipidomics (nonpolar hydrophobic lipids; plasma) and metabolomics (polar hydrophilic compounds; serum, milk, and liver) were performed using C30 and pHILIC columns on a mass spectrometry platform in positive and negative modes, respectively. Statistical analyses for omic data were performed using MetaboAnalyst 4.0 (Chong et al., 2018) following generalized log-transformation and auto-scaling. Data were analyzed using ANOVA and partial least squares discriminant analysis (PLS-DA).

Plasma, milk, and urine TMAO concentrations increased linearly with increasing intravenous dose of TMAO (e.g., 12 to 204 μM in cows infused 0 and 60 g of TMAO/d, respectively; $P < 0.01$). The majority of TMAO was excreted in urine. Dry matter intake was not modified by intravenous TMAO infusion. Milk yield and composition (i.e., fat, protein, and lactose), energy-corrected milk, and feed efficiency were not modified by treatment. Plasma triglyceride, total fatty acid, and glucose concentrations were not modified by TMAO infusion. Serum albumin, total protein, globulin, total bilirubin, aspartate aminotransferase, γ-glutamyl transferase, and glutamate dehydrogenase concentrations were not modified by treatment. However, serum glutamate dehydrogenase concentrations decreased linearly with increasing dose ($P < 0.05$). Changes in circulating glucose or total fatty acids post glucose challenge were not modified by TMAO, which suggest that insulin-stimulated glucose utilization was not modified. Lipidomics analysis revealed 143 plasma lipids. The PLS-DA model

distinguished TMAO treatments; however, only 44 lipids (~31%) were modified by TMAO treatment. No apparent pattern behaviors were observed. Examples of changes were limited to phosphatidylcholines (e.g., PC 36:4 and 37:2 were lower and PC 37:5 was higher in cows infused 60 g of TMAO/d, relative to control; false discover rate < 0.05). Metabolomics analyses revealed 52 serum, 12 milk, and 39 liver compounds with a mzCloud mass spectral score >75%; however, detected metabolites exceeded 100 for each sample type. We were not able to identify unique treatment metabolomes with PLS-DA. In addition, ANOVA did not detect differences in any metabolite with TMAO treatment. We conclude that the acute intravenous infusion of TMAO does not modify measures of liver health, glucose tolerance, or milk production in early lactation cows.

Summary

The scientific discussion on the effects of domestic animal food consumption on cardiovascular disease and NAFLD in humans now includes an emerging debate centered on the role of TMAO. We recognize that increases in circulating TMAO is prognostic and diagnostic of these diseases. In addition, an enhanced TMAO status is attributed to the increased intake of choline, betaine, or L-carnitine from meat, dairy, and eggs, which are converted to TMA and TMAO via the actions of bacterial enzymes in the lower gut and FMO3 in the liver, respectively. However, it is grossly premature to definitively pinpoint TMAO or a dietary TMAO precursor as the cause of these diseases in humans without consideration of the gut microbiota, intestinal barrier functionality, and kidney function. These factors may influence a patients TMAO status and possibly represent the true underlying cause of the disease. In dairy cattle, our investigative work suggests that TMAO does not overtly influence the health status or milk production of the animal; however, lower-gut degradation of choline has the potential to limit choline bioavailability. The extent of this response is not yet defined but should be considered when defining metabolizable choline supply in cows fed rumen-protected choline.

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Update on DCAD for Dry and Lactating Cows

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Introduction

The concept of managing the dietary cation-anion difference for improved health and performance of dairy cattle has existed for more than 30 years (Horst et al., 1997; NRC, 2001). It is well-established that decreasing the dietary cation-anion difference in the diet fed during the prepartum period improves calcium status and decreases risk of hypocalcemia during the immediate postpartum period (Goff, 2014). In addition, a meta-analysis conducted by Hu and Murphy (2004) suggested that increasing the dietary DCAD of diets fed during lactation increased milk yield and dry matter intake (DMI). The focus of this paper is to provide an update regarding the most recently available information related to the application of DCAD in diets for both dry and lactating dairy cows.

DCAD During the Prepartum Period to Decrease Hypocalcemia

As indicated above, decreasing the DCAD [$(Na^+ + K^+) - (Cl^- + S^{-2})$] of the diet fed during the last several weeks before calving decreases hypocalcemia. Several mechanisms are likely responsible for this, including increased calcium flux related to increased urinary calcium excretion and increased sensitivity of tissues to parathyroid hormone for cows fed acidogenic diets (Wilkens et al., 2020). Research from our laboratory demonstrated that further decreasing the DCAD of a low K^+ control diet using anionic supplements linearly increased plasma calcium levels and linearly increased postpartum DMI and milk yield (Leno et al., 2017). Researchers at the University of Florida fed either positive or negative DCAD diets with two different sources of Vitamin D (cholecalciferol or calcidiol) during the prepartum period (Martinez et al., 2018; Rodney et al., 2018). Feeding negative DCAD prepartum increased postpartum circulating concentrations of total and ionized calcium, but did not affect postpartum DMI or milk yield; source of Vitamin D did not affect circulating calcium concentrations, but calcidiol supplementation prepartum increased postpartum milk yield.

Subsequent work by Lopera et al. (2018) sought to determine whether relationships existed between degree of acidification with anions and duration of feeding with outcomes. They fed diets with DCAD of -7 mEq/100 g DM (actual urine pH ~6.5) or -18 mEq/100 g DM (actual urine pH ~ 5.6) for either 21 or 42 d prepartum. Feeding the more negative DCAD diet decreased prepartum DMI, increased blood ionized Ca concentrations on the day of calving, and did not affect postpartum performance. Extending the duration of feeding did not affect blood calcium but decreased milk yield by 2.5 kg/d. These results differ from those of Weich et al. (2013) and Wu et al. (2015) who reported that extending the duration of feeding negative DCAD diets up to 42 d before

calving did not affect postpartum outcomes; however, in both of these studies the urine pH were more similar to the cows fed the -7 mEq/100 g DM diet in the Lopera et al. study.

Recently, two meta-analyses have been published that provide updates to previously conducted meta-analyses exploring the effects of DCAD and other macrominerals on hypocalcemia and performance. Santos et al. (2019) assembled a dataset including 42 experiments with 134 treatment means and 1,803 cows (including 5 experiments with 15 treatment means and 151 nulliparous cows) and evaluated relationships with outcomes. They developed a model to compare the estimated effects of decreasing the DCAD from +20 to -10 mEq/100 g of DM. Using this model, decreasing the DCAD resulted in a predicted decrease in DMI of 0.7 and 0.4 kg/d for nulliparous and parous cows, respectively. An interaction of parity and prepartum DCAD was present such that parous cows fed the negative DCAD produced 1.7 kg/d more milk postpartum; whereas milk production in nulliparous cows was not affected by prepartum DCAD. The more negative prepartum DCAD was predicted to increase postpartum blood Ca, decrease postpartum beta-hydroxybutyrate concentrations, and decrease incidence of milk fever, retained placenta, and metritis.

Lean et al. (2019) assembled a dataset including a maximum of 31 experiments, 58 comparisons, and a total of 1,571 cows with the objective of exploring the effects of reducing DCAD intake on outcomes. Treatments reflecting the lower DCAD intake had lower urine pH, lower DMI, increased postpartum DMI, and increased milk yield, although an interaction for parity existed for milk yield. Consistent with the Santos meta-analysis, treatments reflecting the lower DCAD intake decreased risk for clinical hypocalcemia and retained placenta and lowered the odds of metritis. Both meta-analyses highlighted the relative lack of data on the relationships of prepartum dietary DCAD on outcomes in nulliparous cows.

Dietary Calcium Supplementation with Low DCAD Prepartum Diets

Dietary calcium supplementation strategies in conjunction with low DCAD diets fed during the prepartum period continues to be an active area of discussion and debate in the industry. Moore et al. (2000) reported that concurrently decreasing the DCAD (+15, 0, -15 mEq/100 g) and increasing dietary calcium concentration (0.44, 0.97, 1.50% of DM) improved blood calcium status postpartum; however, the effects of DCAD and calcium supplementation cannot be separated in their experiment. Diehl et al. (2018) fed cows either moderate (~ -2.4 mEq/100 g) or low (~ - 21 mEq/100 g) DCAD diets with either 1.3% or 1.8% calcium during the prepartum period. Few differences in circulating Ca concentrations or performance were observed, except that cows fed 1.8% Ca had higher circulating Ca concentrations at d 1 postpartum and cows fed low DCAD made more milk after 45 DIM.

Recently, Glosson et al. (2020) fed cows either a non-acidogenic positive DCAD diet (+6 mEq/100 g; average urine pH ~ 8.1), or two negative DCAD diets (-24 mEq/100 g; average urine pH ~5.75) fed with either low dietary calcium (0.40% of DM) or high dietary calcium (2.0% of DM) for the last 28 d prior to calving. Feeding negative DCAD

slightly decreased prepartum DMI, increased both ionized and total calcium concentrations in blood directly after calving and 24 h after calving, and increased total calcium concentrations at 48 h postcalving. Postpartum DMI (% of body weight) tended to be increased for cows fed negative DCAD prepartum, but milk production was not affected by treatment.

Finally, Amundson et al. (2018) created an experimental model of hypocalcemia in nonlactating, nonpregnant cows and evaluated three different calcium feeding levels (0.45, 1.13, 2.02% of DM) in conjunction with low DCAD (-18 mEq/100 g DM). Hypocalcemia was induced by intravenous infusion of a Ca-specific chelator, EGTA. Cows fed the highest calcium concentration maintained higher circulating concentrations of ionized calcium during the challenge period, took longer to reach 60% of baseline circulating calcium concentrations, and required more EGTA to reach 60% of baseline concentrations, suggesting that cows fed higher calcium levels.

Increased Dietary DCAD for Lactating Cows

As described above, the meta-analysis conducted by Hu and Murphy (2004) was the first to summarize performance responses of lactating cows in the context of varying DCAD, and suggested curvilinear responses of both DMI and milk yield (along with fat-corrected milk) to increasing dietary DCAD. However, closer examination of most of the experiments included in this meta-analysis reveals that, in many cases, cations were added in the form of compounds with known rumen buffering capacity (e.g., sodium bicarbonate, potassium carbonate, potassium bicarbonate). Furthermore, for many of the low DCAD treatments included in the dataset, the anion concentrations were increased through addition of calcium chloride and ammonium chloride, and do not represent diets that would typically be fed to lactating dairy cows.

Harrison et al. (2012) reported that increasing dietary K from 1.3 to 2.1% of DM using a commercially available potassium carbonate sesquihydrate source increased milk fat percentage and tended to increase fat-corrected milk yield. Evidence in this study for a ruminal effect of treatment is the decreased content of *trans*-10 C18:1 in milk fat, which is correlated negatively with milk fat content (McCarthy et al., 2019). This would suggest a ruminal effect either related to potassium or to the increased buffering provided by the treatment.

Iwaniuk et al. (2015) conducted three experiments to evaluate the effects of cation addition and source of cations in diets for lactating cows. In experiment 1, they added 4, 9, and 13 mEq/100 g of DM from potassium carbonate to a basal diet containing +16 mEq/100 g of DM [$(Na^+ + K^+) - (Cl^- + S^{-2})$]. Neither milk yield nor DMI were affected by treatment; however, feeding increasing amounts of potassium carbonate linearly increased milk fat percentage and yield, and increased yields of 3.5% fat-corrected milk. In experiment 2, they added 11, 23, and 35 mEq/100 g of DM from potassium carbonate to a basal diet containing +19 mEq/100 g of DM. Dry matter intake was increased linearly by increasing amounts of potassium carbonate. Milk yield as not affected by treatment, but again potassium carbonate supplementation linearly increased milk fat percentage

and quadratically increased yields of milk fat and 3.5% fat-corrected milk. In experiment 3, they fed four diets with very similar DCAD, but varied the proportions of potassium and sodium by varying the proportions of potassium carbonate sesquihydrate and sodium sesquicarbonate in the diet. Neither DMI nor milk yield were affected by treatment, but as cows were fed more sodium sesquicarbonate, milk fat percentage and yield were increased linearly.

Catterton and Erdman (2016) fed lactating cows a basal diet of +20 mEq/100 g DM or diets supplemented with about 34 mEq/100 g of Na from NaCl, 34 mEq/100 g of K from KCl, 34 mEq/100 g from sodium bicarbonate, or 34 mEq/100 g from potassium carbonate, resulting in calculated DCAD for the supplemented diets of 20, 19, 54, and 54 mEq/100 g, respectively. There was a significant effect of DCAD on rumen pH such that cows fed sodium bicarbonate and potassium carbonate had higher rumen pH than the other three treatments, and an effect of anion in that cows fed KCl and NaCl had lower rumen pH than those fed bicarbonate or carbonate.

In summary, the original meta-analysis conducted by Hu and Murphy (2004) characterized relationships of DCAD with performance outcomes, it appears that relationships observed are likely to be the consequence of supplementation of sodium and potassium sources that also have a buffering role in moderating or increasing rumen pH, although work in continuous culture fermenters by Jenkins et al. (2014) suggests that there may be an effect on ruminal fermentation of potassium independent of ruminal pH. The studies with very low DCAD that are primarily responsible for the curvilinear response surfaces had high levels of anion supplementation that are not representative of diets fed to lactating dairy cattle.

Summary

Decreasing the DCAD of the prepartum diet fed to dairy cattle is effective at improving postpartum calcium status as well as milk yield in general. Recent work suggests that feeding lower DCAD levels that result in urine pH values around 5.5 may be detrimental if continued beyond the typical 21 to 28 day close-up period. Questions remain around how aggressive nutritionists should be in their DCAD and urine pH targets as well as appropriate dietary calcium supplementation levels. Recent work in model systems suggest that higher dietary calcium in the context of low DCAD diets may result in improved calcium status. Effects of sodium and potassium supplementation in lactating cow diets have long been rationalized in the context DCAD; however, the available information suggests that it is much more likely that the sources fed (i.e., carbonate, bicarbonate, or sesquicarbonate) are having effects as rumen buffers rather than a specific effect on postabsorptive acid-base balance, although there may also be a role for potassium in rumen microbial fermentation.

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Milk Urea Nitrogen: Precision, Accuracy, and Individual Animal Variability

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Introduction

Nitrogen loss from dairy farms negatively impacts the environment by contributing to greenhouse gas emissions, soil acidification, ground water contamination and surface water eutrophication (Hristov et al. 2011). Much of the N lost from dairy farms originates from manure N and, in particular, urinary N. Thus, reducing the amount of nitrogen excreted by individual cows is an important step in reducing the detrimental environmental effects of the dairy industry. Nutrition strategies that increase the efficiency with which feed N is converted into milk N reduce the amount of manure N produced per unit of milk. Milk Urea Nitrogen (MUN) is commonly used as an indicator of protein metabolism and nitrogen efficiency in lactating dairy cattle to guide management and diet formulation decisions.

Urea is a byproduct of protein metabolism generated when the liver converts the ammonia produced during amino acid catabolism into non-toxic urea that is primarily excreted in urine. Due to the ability of urea to diffuse and equilibrate across membranes that separate blood, urine, and milk, plasma urea N (PUN), MUN, and urinary urea N (UUN) concentrations are highly correlated such that one measurement can be used to estimate the others (Gustafsson and Palmquist, 1993). Since milk sampling already occurs regularly on farms and is less invasive than blood sampling, MUN is the preferred method for estimating UUN excretion and assessing N efficiency on commercial dairies.

Unfortunately, previous research raised uncertainty about the precision and accuracy of commercial MUN testing, suggesting that results from a the same set of bulk tank samples sent to multiple labs had a wider range of reported results than the recommended target range of 8-12 mg/dl (Weeks and Hristov, 2017). Thus, one objective of this study is to reevaluate the precision and accuracy of mid-infrared spectroscopy (MIR) for MUN analysis. Additionally, accurate interpretation of MUN values requires an understanding of natural variation in MUN over time. While dairy farms often measure MUN during routine bulk tank sampling, the movement towards precision management will benefit from individual cow and pen-level metrics like MUN. Therefore, the secondary objective of this study is to quantify the expected variation in MUN over the course of lactation in individual cows. Combination of testing precision and expected variation will enable more effective interpretation of MUN results.

Methods

Precision and Accuracy of MIR MUN Analysis

Bulk tank samples were collected for 7 consecutive days and sent to 3 commercial labs (Labs A, B, and C) and the Barbano Lab (Lab D) in duplicate. Bulk tank samples were collected daily between 10:00 and 13:00. Samples were immediately placed on ice, stored overnight at 40 ^{o}F, and either delivered the following morning to Labs A and D or shipped to the additional commercial labs (Labs B and C).

Additional sample sets from the Federal Milk Market Administrator (FMMA) quality assurance program were prepared by Lab D and sent out to Labs A, B, and C for MUN analysis. The FFMA quality assurance program prepares 10 milk samples every 2 weeks from around the country that are composite samples from multiple bulk tanks in that region. The sets are used to ensure the accuracy of milk testing labs meet the USDA's standards for milk payments which are based on milk fat and protein content. MUN is not included in this quality assurance program so labs are not required to report their MUN results from these sample sets. Lab D prepared three sets of the FFMA samples in duplicate (i.e. 20 samples each) on three separate weeks for shipment to Labs A, B, and C.

Mid-infrared spectroscopy was used to evaluate MUN content of all samples at each lab. In addition, Lab D performed the Megazyme Urea/Ammonia Assay Procedure (Barbano and Coon, 2017) on all samples and this enzymatic assay was used as the reference chemistry for subsequent data analysis and comparison.

Individual Cow Variation

Milk samples were collected from 16 multiparous Holstein cows at each of 3X daily milkings during 3, 7-day periods in early, middle, and late-lactation. Samples were collected in triplicate in period 1 (P1) and in duplicate for periods 2 and 3 (P2 and P3). One set was sent to commercial Lab A for MIR analysis of MUN and milk components. Lab D analyzed the second set with MIR analysis for milk components and MUN and performed the enzymatic assay for MUN on the third sample set collected for P1.

Animal Care and Sample Collection

Animals were housed at the Cornell University Ruminant Center and all procedures were reviewed and approved by the university's IACUC. During P1, cows were housed in tie stalls. In P2 and P3 cows were moved to pens and were required to be in the pen for a minimum of one-week prior to each sampling period. Two of the 16 cows were culled during the study period leaving 14 cows that made it through the each of the 3 periods.

Cows were fed a standard high-cow TMR with an average CP content of 15.4% that ranged from 14.7-16.2%.

Milk samples were collected from individual cows using DeLaval in-line sampler and production, time and date were recorded for each sample. All samples were transferred to 1 L plastic bottles, inverted to mix, and aliquoted into sub-sample tubes for Labs A and D. Samples were stored at 40 ^{o}F before delivery to Labs A and D. Samples collected for MIR analysis were processed immediately, while samples to be used in the enzymatic MUN assay were frozen and stored for later processing.

For all samples, MUN was measured using MIR technology. Lab A analyzed samples using Milkoscan FT+ and Milksocan FOSS 7 spectrometers while Lab D used the Delta FTA. Both labs reported values for milk fat, protein, lactose, somatic cell count (SCC), and MUN.

Statistical Analysis

All data analysis was performed in R version 3.6.3. Mixed models were fit with the lmer() package and all other functions were performed using base packages.

Precision and Accuracy of MIR MUN

Statistical methods for evaluation of methodological agreement described by Lynch (1998) and used by Kaylegian et al. (2006) were applied to bulk tank MIR MUN analysis results. The Mean Difference (MD) was calculated by subtracting the reference chemistry value from the MIR spectroscopy value and averaging the difference over the sample sets. The standard deviation of the difference (SDD) was calculated as the square root of the summed squared value of the differences divided by the number of samples. The Euclidian distance (ED) was calculated as the distance from the origin of the points when the SDD is plotted against the MD. The coefficient of variation (CV) was calculated as the SDD divided by the mean reference chemistry MUN for each sample set. The repeatability (sr) was estimated for each commercial lab by calculating the square-root of the summed squared differences between duplicate analyses on the same sample divided by the number of samples. The reproducibility (sR) was also estimated for all labs by calculating the square-root of the summed, squared differences between the MIR analysis and the reference chemistry divided by the total number of samples tested at each lab.

Sample resutls were also fit to a linear mixed model:

$$\mathrm{MUNDiff}_{ij} = \beta_0 + \beta_1 \mathrm{MUNRefC}_{ij} + \beta_2 \mathrm{Prot}_{ij} + \beta_3 \mathrm{Fat}_{ij} + \lambda_i + \sigma_{ij} \quad [1]$$

In Eq. [1], $\mathrm{MUNDiff}_{ij}$ is the difference between the MIR analytical value for MUN and the reference chemistry MUN for *j*th sample from the *i*th lab; β_0 is the intercept that represents the mean difference between the reference chemistry and the MIR analysis; β_1 is the slope that represents the change in MUNDiff as the reference chemistry MUN value moves away from the mean of the reported values; $\mathrm{MUNRefC}_{ij}$ is the mean-centered value of the MUN reference chemistry, Prot_{ij} and Fat_{ij} are the MIR values for the true protein and fat composition and β_2 and β_3 are the slopes that represent the change

in MUNDiff as milk protein and fat increase, respectively; λ_i is the random effect of the *i*th lab; and σ_{ij} is the residual random error.

Individual Animal MUN Variation

The individual animal MUN data from all three periods was fit to the following models:

$$\mathrm{MUN}_{ijklm} = \mathrm{Lab}_i + \mathrm{Milking}_j + \mathrm{Period}_k + \alpha_l + \delta_m + \sigma_{ijklm} \quad [2]$$

$$\mathrm{MUN}_{ijklm} = \mathrm{Lab}_i + \mathrm{Milking}_j + \mathrm{Period}_k + \beta_{1i}\mathrm{Fat}_{ijklm} + \beta_{2i}\mathrm{Protein}_{ijklm} + \alpha_l + \delta_m + \sigma_{ijklm} \quad [3]$$

In Eq. [2] MUN_{ijklm} represents the raw MUN value for a sample tested by the *i*th Lab (labs A or D), collected during the *j*th milking of the *k*th period from the *l*th animal on the *m*th date. Lab was included as a fixed effect rather than fitting a model to each lab separately. Neither DIM nor CP level are included as variables due to a high correlation between these two potential variables as a result of the short sampling periods. Instead, period is included as a variable, because the effect of period is related to the stage in lactation and accounts for external factors including weather, pen, etc.

In Eq. [3], the dependent variable is the same but the fixed effects include parameters to estimate the effects of fat and protein content on reported MUN concentration. In Eq. [3], β_{1i} is the effect of milk fat corresponding to the *i*th lab and β_{2i} is the effect of milk protein corresponding to the *i*th lab. These parameters are included because the bulk tank analysis showed that fat and protein content impacted the MIR difference from enzymatic MUN measurements. The parameters act as a correction factor and should therefore remove any effect cause by fat and protein interference with MIR analysis.

In both models, α_l is the random effect of the *l*th animal, δ_m is the random effect of the *m*th day, and σ is the residual random error

Results and Discussion

Precision and Accuracy of MIR MUN Analysis

The plot of the SDD vs. MD is presented in Figure 1 and the ED, which are not significantly different between labs, are presented in Table 1. There is no apparent pattern or grouping in the plot in Figure 1 which suggests that there is no systematic bias in MUN reporting for the labs included in this study. The only potential pattern that emerges is that the points from the first machine in Lab D (D1), all fall in the negative range of the x-axis (to the left of the vertical line at MD=0) which suggests that the MIR results from this machine within this lab tend to under estimate MUN.

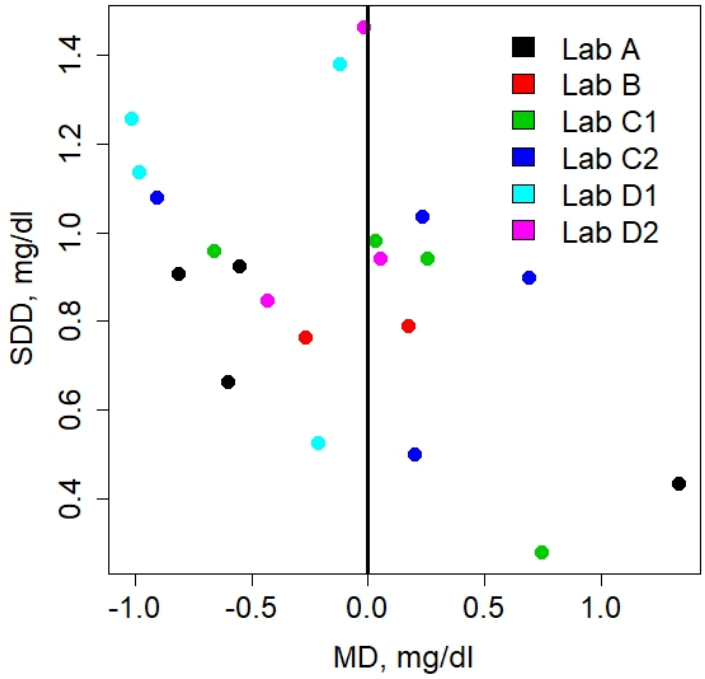

Figure 1. A plot of the standard deviation of the difference (SDD) vs mean difference (MD) for each lab (A-D) with labs C and D reporting results for two different machines.

Table 1. Euclidean Distance of MIR analysis of MUN for Labs A-D. Labs C and D reported results for two different machines which is indicated by the label.

Lab	Euclidean Distance
A	1.15
B	0.810
C2	0.978
C1	1.04
D1	1.27
D2	1.12

Repeatability (s_r) and reproducibility (sR) estimates for commercial labs are shown in Table 2 and ranged from (0.297 - 0.469) and (0.555-0.791) respectively. Repeatability is interpreted as the expected variability of a result reproduced by the same lab on the same sample. Similarly, reproducibility is the expected difference or variation between two labs or methods. In this analysis, reproducibility measures the ability of commercial lab MIR to reproduce the enzymatic assay. Repeatability and reproducibility values are interpreted like standard deviations. Since all sR values are less than 1, each of the labs is expected to predict MUN within 0.8 mg/dL of the reference chemistry value 68% of the time. This means that 95% of the time, each lab is expected to predict MUN within ± 1.6 mg/dL. Looking at the repeatability measures, all labs have an sr value < 0.5 mg/dl. This means that 95% of repeated sampling is expected to be within ±1 mg/dl. These parameters can also be expressed as percentages, similar to a coefficient of variation,

which indicate the percent of the mean MUN value by which repeated and reproduced analyses are expected to vary.

Table 2. Repeatability and reproducibility values and percentages for the commercial labs included in this study.

Lab	sr (mg/dL)	sr (%)	sR (mg/dL)	sR (%)
A	0.367	2.98	0.785	6.38
B	0.362	2.94	0.555	4.51
C1	0.297	2.41	0.701	5.70
C2	0.469	3.81	0.791	6.43

Differences in sr and sR across labs are most likely due to the use of different machines and calibration methods used for different machines.

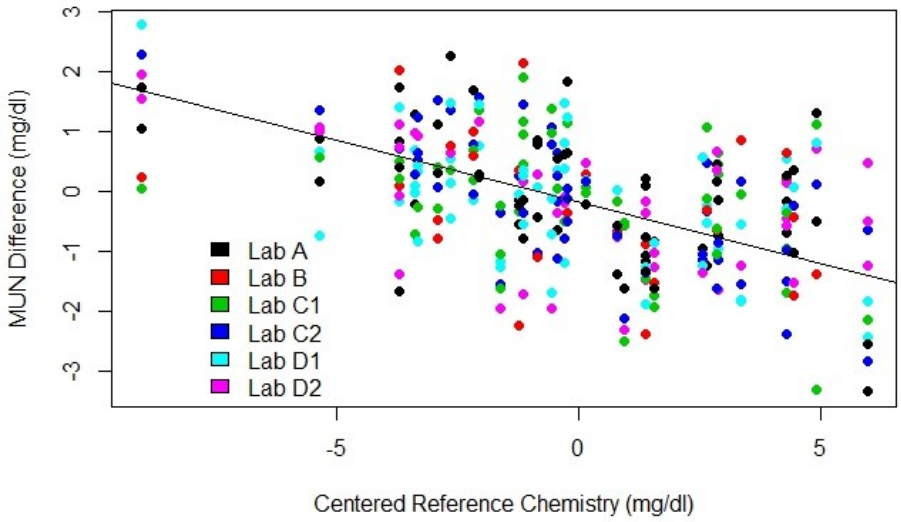

Figure 2. A plot of the differences between the MIR and reference chemistry for MUN analysis vs. the centered reference chemistry value. Line represents the fixed-effect results of the mixed-model regression.

Regression results indicate MIR analysis over-predicts MUN at low MUN concentrations and under predicts MUN at high MUN concentrations. A plot of the MUN differences against the centered reference chemistry values is shown in Figure 2 and the parameter estimates are provided in Table 3.

Table 3. The parameter estimates from a mixed-model analysis described in Eq. [1]

Parameter	Mean	SE
β_0	-2.32	0.436
β_1	-0.206	0.0168
β_2	0.397	0.1629
β_3	0.193	0.0893
σ_{Lab}	NA	0.224
σ_{Res}	NA	0.868

The results of the mixed-model analysis suggest that at the mean milk protein (3.4%), milk fat (4.2%), and MUN (12.8 mg/dl) of this dataset, MIR analysis was not significantly different than the reference chemistry. However, for every 1 mg increase in the reference chemistry value (what is considered to be the true MUN value) above 12.8 mg/dl, MIR analysis underpredicted MUN by an average of 0.206 mg/dl. This means that for a milk sample with 3.4% protein, 4.2% fat, and 15.8 mg/dl MUN, MIR analysis would be expected to underpredict MUN by 0.618 mg/dl. Similarly, as the reference chemistry decreases below the average of 12.8 mg/dl, the MIR analysis is expected to over predict MUN concentration. For a milk sample with 3.4% protein, 4.2% fat, and 7.8 mg/dl MUN, MIR analysis would be expected to over predict MUN by 1.3 mg/dl. In addition, as protein and fat levels deviate from the means in this dataset, MIR MUN analysis is expected to have some systematic over or under prediction depending on the linear combination of the fat and protein levels and their parameter estimate.

The residual standard error estimate (0.87 mg/dl) and random effect of lab (0.22 mg/dl) indicate the amount of uncertainty in MIR analysis of MUN. Combining these variance estimates, we get an overall standard error of 0.90 for MUNDiff which means at the average milk composition values, the 95% CI for differences between the MIR analysis and the reference chemistry to be between -1.8 and +1.8 mg/dl, which is very similar to the results of the reproducibility analysis reported above.

Individual Cow Variability

The mean and SE of the regression parameters corresponding to Eq. [3] and [4] are listed in Tables 4 and 5. A separate intercept representing the average MUN value during Period 1 and Milking 1 was estimated for Labs A and D. For example, for Eq.[2] the Lab_A parameter estimate is the average value for Lab A at milking 1 in Period 1, and in Eq.[3], the Lab_A parameter estimate represents the expected MUN value at milking 1 in Period 1 at a fat and protein content of zero. In both models, the fixed effects estimates indicate the amount by which MUN is expected to increase or decrease based on milking time and period of lactation. For example, in Eq. [2] milk samples collected during milking 1 in Period 2 are expected to have MUN values 0.391 mg/dL less than samples collected during milking 1 in P1. Likewise, samples collected during milking 3 of P3 are expected to have an average net difference in MUN values of 0.472 mg/dL compared to samples taken during milking 1 of P1.

The proportion of variance caused by the random effects for animal and date are similar between the two equations and indicate that approximately 30% of the variation is attributable to individual animals and only about 8% of is explained by variation from day to day. An additional 62% of total variance is attributed to residual random error that cannot be explained by either model but contains the variation associated with lab repeatability. The total variance including the random effect of animal, day, and residual error is 3.667 which equates to a standard error of MUN of 1.91 mg/dL. Thus, the random variation associated with MUN observations over multiple days would be expected to be within ± ~ 4 mg/dl. Removing the random effect of animal, the expected MUN variance of an individual animal across multiple days is 2.55 or a SE of 1.6 mg/dl. From a management perspective, observations varying more than ± 1.91 mg/dL for multiple

animals in a pen or 1.6 mg/dl for a single animal between days indicate that a significant change has taken place. For example, a MUN value of 10 mg/dL one week, followed by a diet change and an MUN of 9 mg/dL the following week, may indicate that the diet change had no significant impact on MUN.

Table 4. Parameter estimates of linear mixed model for the effects of lab, milking time, and period of lactation as described in Eq.[2]

	Estimate	SE	σ	Prop. of variance
Lab_A	7.90	0.349		
Lab_D	7.03	0.349		
$Milking_2$	0.289	0.0877		
$Milking_3$	-0.307	0.0886		
$Period_2$	-0.391	0.314		
$Period_3$	0.779	0.3151		
α		1.05	1.124	30%
δ		0.565	0.327	8.9%
σ		1.54	2.220	61.1%

Table 5. Parameter estimates of linear mixed model for the effects of lab, milking time, period of lactation, milk fat, and milk protein as described in Eq.[3]

	Estimate	SE	σ	Prop. of variance
Lab_A	10.5	0.797		
Lab_D	5.63	0.762		
$Milking_2$	0.351	0.0919		
$Milking_3$	-0.239	0.0934		
$Period_2$	-0.332	0.352		
$Period_3$	0.783	0.366		
β_{1A}	0.140	0.0980		
β_{1B}	-0.599	0.1180		
β_{2A}	-1.08	0.2702		
β_{2B}	2.15	0.196		
α		1.06	1.124	30%
δ		0.572	0.327	8.9%
σ		1.49	2.220	61.1%

Summary

In order to interpret reported MUN values, the precision and accuracy of the metric must be taken into account. The results presented here suggest that MIR analysis of MUN has improved since the 2017 report by Weeks and Hristov as the commercial labs that participated in this study were able to reproduce results of the enzymatic assay within ± 1.6 mg/dl. Further, commercial lab repeatability of MUN was high. However, the systematic bias revealed by the regression analysis indicates that there is still a need for improvement in MIR methods for this important milk component. Further, if MUN is to be used as a metric for management of individual animals, the metric must also be interpreted within the context of that animal's natural variation. Removing the MUN variation between animals, we found that the MUN of an individual cow would be expected to vary ± 1.6 mg/dL from day to day under similar dietary conditions and lactation period.

Take Home Messages

- Commercial lab repeatability for MIR analysis of MUN is ≤ 0.5 mg/dl which is lower than 5% of the average MUN value
- Commercial lab MIR reproducibility of the gold standard method for measuring MUN is ≤ 0.8 mg/dl which means reported MIR values are expected to be within 1.6 mg/dl of the true value
- Current MIR methods for analysis of MUN tend to over predict MUN at values below 12.8 mg/dl and under predict MUN at values above 12.8 mg/dl
- The MUN content of samples from an individual cow is varies between days so repeated samples from the same cow across multiple days within the same stage of lactation would be expected to vary ± 1.6 mg/dL

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Reducing Feed Costs While Maintaining Milk and Milk Component Production

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Dairy producers have been challenged with low milk prices and decreased profitability in the last few years. This has provided the opportunity to examine and evaluate their herd management system to define changes that could be made to lower costs without impairing milk production. On most dairy farms, feed cost is the largest single item in determining the cost of milk production. Feed costs may account for 35 - >45% of the total cost of producing milk. Potential adjustments in the feeding program when milk prices are low have been the subject of previous papers (Chandler, 2003; Weiss and St-Pierre, 2013).

Hutjens (2010) reported the results of a survey of nutritionists, veterinarians and extension educators on management changes made in response to low milk prices in 2009. The top 5 correct decisions made were emphasis on the forage program, staying the course with management practices, reviewing ration balancing and the nutrition program, strategic culling and paying attention to milk components and quality. The top 5 incorrect decisions were reducing feed intake or removing nutrients, taking out minerals, vitamins and feed additives, not staying the course, low forage quality and avoiding financial support.

This situation provides dairy producers an opportunity to examine current herd nutrition and management practices. This evaluation should be done in cooperation with the herd nutritionist and other consultants working with the herd. The goal is to explore potential changes that could lower feed costs without impairing milk production, herd health or reproduction. Don't make short-term changes to lower feed costs that could have a long-term negative impact on herd performance and profitability. The transition cow program is one example. The results of this program directly impact metabolic disorders, peak and total lactation milk and reproduction.

Take Advantage of Home-Grown Forages

The forage program on a dairy farm is the foundation for developing successful, efficient, healthy and profitable feeding programs. Forage quality, consistency, inventory and allocation are the key areas. The goal is to provide the "right" quality forage to match nutrient needs of the various groups on the farm. Table 1 contains information on 4 forages available on a specific farm. In terms of matching cow requirements, corn silage B and haylage B would fit best for early lactation, high producing cows. Corn silage A and haylage A would better match the nutrient needs of later lactation cows and bred heifers.

Table 1. Forage Quality of Available Forages

Item	Corn Silage A	Corn Silage B	Haylage A	Haylage B
Dry matter, %	32.4	34.5	36	35.8
CP, % of DM	7.8	7.2	15.3	21.1
ADF, % of DM	31	23.1	37.3	26.8
NDF, % of DM	45.4	38	52.8	36.3
NDFD, 30-hour, % of NDF	50.7	59.8	48.1	57.2
Starch, % of DM	28.5	38	-	-

A simple way determined the amount of forage to include in the ration is to use forage NDF intake. A good starting point is forage NDF intake as 0.9 to 1% of body weight. This can then be adjusted based on forage NDF and NDFD values. A more recent method is to use $uNDF_{240}$ in the total ration as 0.3 – 0.4% of body weight for herds feeding corn silage-based rations. A paper from Italy reported total ration $uNDF_{240}$ intakes of 0.4 to 0.48% of body weight for alfalfa hay and chopped wheat straw diets (Fustini et. al., 2017)

What levels of forage are currently fed in dairy rations? A dataset of 16 Holstein herds or groups with an average MP predicted milk of 109 pounds was extracted from a previous paper Chase, 2019). The average ration forage level was 64.3% with a range of 60 to 72%. Total ration NDF was 30.8% (range of 26 to 34%) and forage NDF intake as a percent of body weight of 0.95% (range of 0.75 to 1.14%). A New York herd increased ration forage from 55% of the ration dry matter to 65% over a 4-month period. This was done by the herd nutritionist as a result of improvements in forage quality. Forage NDF intake increased from 0.84 to 0.96% of body weight. Milk production increased by about 2 pounds per cow. The income over purchased feed cost increased by $1.30 per cow per day.

Forage inventory is a key consideration when moving to higher levels of forage feeding. It may take 15 – 30% more forage to feed the same number of cows. Make sure to do a forage inventory to assure that adequate quantities of forage are available before implementing a higher forage feeding program.

Feed Additives

There are several feed additives that can be used in dairy rations. The daily cost of using these can range from about 2 to >50 cents/cow/day. A mistake made in 2009 was to remove feed additives from rations without looking at return on investment. It is logical to work with your herd nutritionist to evaluate the feed additives used as part of a decision-making process. Key considerations are:

1. Why was the feed additive originally added to the ration?
2. Is it working in your herd?
3. What is the return on investment?
4. Which group(s) of cows should this additive be targeted to?

5. Is your grouping structure designed to feed targeted additives to only specific groups?

Feed Ingredient Selection

There are many feeds and forages available for use in dairy rations. Many of these are co-products of processing grains for human food. There may be opportunities to select feed ingredients to help in controlling feed costs. The following points should be considered as part of the decision-making process when evaluating feed ingredient choices:

1. Compare choices on cost per unit of nutrient provided rather than cost per ton. A feed may have a low cost per ton but is not a good buy if it does not provide the nutrient(s) needed. Corn gluten feed may be attractively priced but is not a good source of rumen undegradable protein (RUP).
2. Feeds with high RUP will be undervalued unless the program used gives credit for RUP.
3. Try to select feeds with lower levels of variability in nutrient profile. Many co-product ingredients have significant variability due to differences in processing methods and conditions. Many forage testing labs have online feed composition libraries that can be accessed and used to examine the variability of ingredients.
4. When possible, select feed ingredients that come with a feed tag guarantee. There are some co-product blends that provide this information.
5. Another option is to always source feed ingredients from the same processing plant. There should be less variation in nutrient profile within plant than between plants for the same feed ingredient.
6. Using a number feed ingredients in the ration at lower levels of inclusion reduces the impact of nutrient profile of an individual ingredient on potential changes in total ration nutrient composition.
7. Take advantage of cash discounts.
8. Explore options to lock in feed ingredient purchases.

Grouping Considerations

The goal of grouping dairy cows is to increase the uniformity of group by decreasing the variability of milk production within the group (Weiss, 2018). This provides an opportunity to improve production, feed efficiency and improve income over feed costs. A survey in Pennsylvania dairy herds found that 48.8% of the herds fed 1 total mixed ration to the lactating dairy cows (Buza and Holden, 2015). A survey reported that 64% of Michigan herds ≥200 cows fed 2 or more rations to the milking cows (Contreras-Govea et.al., 2015). In the same survey, 69% of the herds in Wisconsin fed 2 or more ration the milking cows. The ability to have fresh cow or first lactation heifer groups were the most common reasons for feeding more than one ration.

A comparison was done to evaluate the changes in income over feed cost (IOFC) on 30 Wisconsin dairy farms (Cabrera et. al., 2012). The IOFC with no grouping was $2,311 per cow per year. The IOFC when 3 feeding groups were used was $ 2,707 for an average increase of $396 per cow. Using 3 feeding groups increased IOFC by $161 per cow in herds < 200 cows and $580 per cow in herds > 1,000 cows. A recent observational study in a 600 cow New York herd found a decrease in total feed cost of $184 per cow per year and $171 in purchased feed cost when a late lactation ration was implemented. This is compared to the herd having fresh cow and milk cow rations before the third group was added. There was no change in milk production in this herd.

There are also options that can help in controlling feed costs in component fed herds. One is to use a 2-grain feeding system. The most common application of this is to have energy and protein grain mixes. The proportions fed to individual cows can be varied to better meet nutritional requirements. An additional approach is to formulate a fresh cow pack. This can contain some feed additives targeted only for fresh cows. Feeding the grain 3-4 times per day will help to maintain a more consistent rumen fermentation

Feed Shrink

Shrink is the feed produced on the farm or purchased that is not consumed by the cow. A 1 percent change in dry matter total mixed ration shrink has been estimated to be $25,000 per year in 1,000 cow herd (Stone et. al., 2015). This is based on 52 pounds of dry matter intake and a feed cost of 13 cents per pound of dry matter. Shrink also changes the cost per ton of feed ingredients (Greene, 2014). A 5% shrink loss in soybean meal purchased at $350 per ton increases the cost to $368 when included in the ration.

Dry feeds stored in flat storage ranged from 3.5 to 13% shrink (Greene, 2014). In the same survey, shrink was 1.5 to 7% when dry feeds were stored in upright bins. This decrease in shrink when bulk tanks were used are like a previous repot (Kertz, 1998). One change taking place on dairy farms is a move towards using upright buns or enclosed commodity barns.

Dry matter losses in bunker silos were reported to be as high as 31% in a review paper (Borreani et. a., 2018). This is for silos with no cover and poor management. Greene (2014) reported shrink values of 9 to 16% for corn silage and 12 to 18% for haylage using observations from commercial herds. Forage storge losses include field and harvest loss, transport loss, storage loss and feed out loss. It is difficult to quantify these losses on an individual farm. Attention to details such as forage dry matter at harvest, rate of filling, packing, sealing with a cover, use of inoculants, feeding out 6 – 12” per day and maintaining a straight, tight face at feed out can have a significant impact on decreasing silage losses and quality.

Replacement Heifers

Feed costs may represent 50 – 60% of the total cost of raising a heifer. As a result of improved reproduction and overall herd management, many herds are raising more heifers than needed if the herd is not expanding. The use of sexed semen can also contribute to a higher number of heifers on the farm. The number of replacement heifers needed can be determined using the turnover rate of the dairy herd, the heifer non-completion rate and the age of heifers at first calving. A 100-cow herd with a 34% culling rate, an 10 % heifer non-completion rate and 24 months at age a first calving needs 76 heifers. If age at first calving is 22 months, the herd needs 69 heifers. One tool that can be used to determine the number of heifers needed in the Heifer Calculator (2020). Genomic testing could be used to determine which heifers to sell.

Ration Formulation

This area offers opportunities for controlling feed costs but requires a team effort with the herd nutritionist to approach this logically to prevent decreases in milk production or milk components. The goal is to provide a balanced ration that optimizes rumen fermentation and microbial protein production (MPP). An opportunity area in many herds is adjusting feed carbohydrate and degradable protein levels to increase MPP. This decreases the amount of more expensive RUP feeds that need to be used in the ration. Microbial protein is also an excellent source of amino acids and may decrease the amount of rumen protected amino acid needed in the ration. Key considerations in going through this process are:

1. Characterize the current ration in terms of feed costs, milk income, income over feed cost (IOFC) and income over purchased feed cost IOPFC). This sets the benchmark for evaluating the potential impact of changes.
2. Use a computer ration model that predicts changes in MPP, milk production, IOFC and IOPFC. Use energy corrected milk (ECM) as the index of changes in milk production.
3. Use metabolizable energy (ME) and metabolizable protein (MP) to evaluate rations.
4. Balance ME and MP as close to requirement as you are comfortable with. Many high producing herds can balance these at < 110% of requirement. This will require providing adequate rumen fermentable carbohydrates and degradable protein as the building blocks.
5. Target MPP as > 50% of the total ration MP. This decreases the quantity of high RUP feeds and rumen protected amino acids that need to be purchased.
6. Balance lysine and methionine using the guidelines from the specific program you are using. Check the other amino acids (especially histidine) to determine how close they are to requirements. If another amino acid is low, the expected response to lysine and methionine may not be observed.
7. Check the ratio of urinary N excretion and the N excreted in milk. A goal is a 1:1 ratio. If the ratio is > 1:1, there may be an opportunity to lower ration crude

protein and decrease nitrogen excretion to the environment and lower ammonia emissions.

Feeding Management

The key to an efficient and profitable ration is consistency in daily management (DeVries, 2019). This encompasses all aspects of the nutrition program. Key questions in this area are:

1. Do cows always have access to a fresh and palatable TMR when in the cow housing area?
2. Are records kept of the amount of each feed added to the TMR? Electronic feed management systems can provide this information?
3. How consistent is the TMR delivered along the length of the bunk? One way to check this is the use of a forage particle separator.
4. Is feed delivered to each group at the same time each day?
5. Can all cows eat at the same time?
6. What do the feed refusals look like? If sorted and mainly coarse particles, the cows were underfed.
7. What is the quantity of feed refusals? This will vary between herds but a target of 1 – 3% can work in some herds.
8. How often is the ration fed and how often are feeds pushed up? Herds with more frequent push-ups tend to lower the variation in the feed consumed and provide more opportunity for increasing dry matter intake and milk production.

Summary

The current situation with milk price provides an opportunity for dairy producers to evaluate areas on the farm that may help in lowering feed cost while maintaining milk and milk component production. It is critical to not decrease milk and milk income in the process. It is also important to not make short-term decreases in feed cost that could have long-term implications on milk production, herd health or reproductive performance. The herd nutritionist and other key advisors need to be part of the team effort to go through an analytical process before any changes in feed cost are made. On many herds, there will be some opportunities to plan in feed costs while maintaining milk income. This will help improve profitsbility.

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