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Could Your Trace Mineral Program Be Doing More Harm Than Good?

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Introduction

Although trace minerals are one of the smallest components of the diet, they are also one of the most important. Copper, zinc, and manganese are vital to animal health and well-being due to their involvement in immunity, fertility, metabolism, and production. Trace mineral supplementation started with oxide forms in the 1930s and moved to the more available sulfate forms in the 1950s when World War II technology advances allowed for more efficient sulfate production. To further improve bioavailability and effectiveness, organic trace minerals were introduced in the 1970s. While many organic trace minerals delivered improved results, their high cost meant producers only replaced a small fraction of the animal's total trace mineral requirement. Hydroxy trace minerals (IntelliBond®) were developed in the 1990s as a more cost-effective improved trace mineral source. The differences in trace mineral sources and their effectiveness mainly lie in the type of chemical bond that binds the metal to its ligand (Figure 1). Sulfate trace minerals contain a metal ion bound to a sulfate ion via an ionic bond. This ionic bond breaks apart easily in an aqueous environment, releasing a free metal ion at liberty to interact with other nutrients or microbes in the rumen. Organic and IntelliBond trace minerals contain stronger covalent bonds that protect the metal from being released too early in the feed or digestive tract, giving them an advantage in diet stability, palatability, digestibility, and bioavailability over sulfate trace minerals.

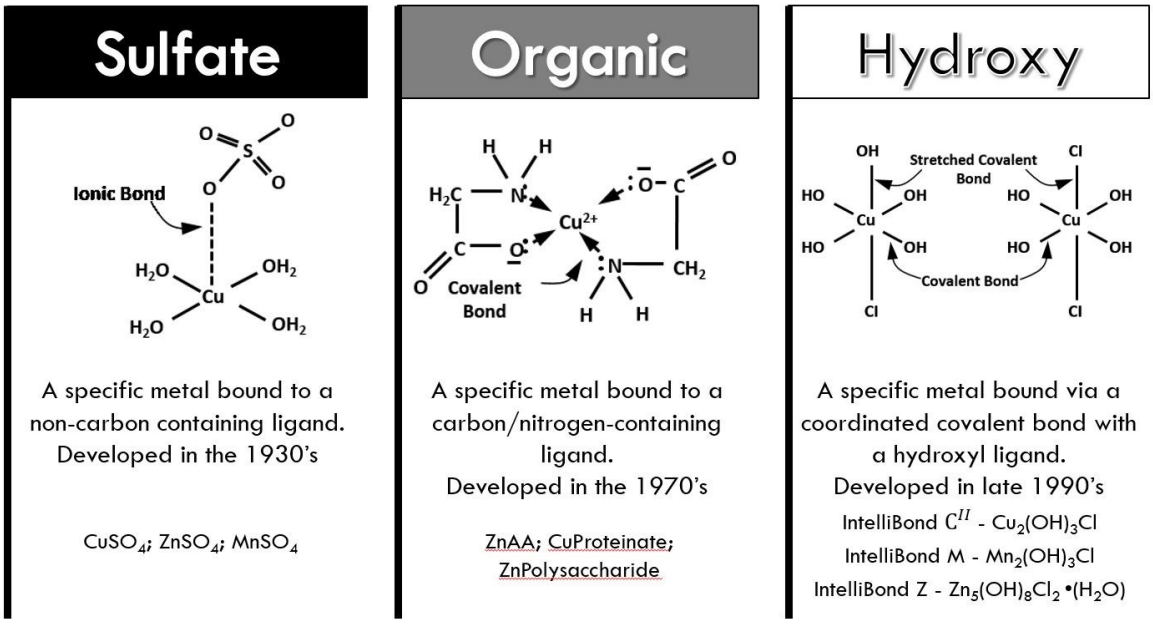


Figure 1. Chemical characteristics of different trace mineral sources

Feed Stability

Some nutrients and additives included in a complete ration may be susceptible to oxidative free metals released by sulfate trace minerals. Vitamins are sensitive to their environment because they are comprised of unsaturated carbon atoms and double bonds vulnerable to oxidation. Coelho (2002) reported on a variety of ruminant premix processing protocols and found that vitamin stability is often compromised when trace minerals were included in the premix. Furthermore, when sulfate or free metals were included in the premix, stability of vitamins A, E, and K were decreased to a greater extent compared to chelated (organic) or oxide trace minerals, indicating that trace mineral source impacted vitamin availability. Research with IntelliBond copper has shown 10-70% more vitamin E retention in complete poultry diets containing 200 ppm copper versus copper sulfate after 10-40 days of storage (Lu et al., 2010; Figure 2), which later corresponded to higher liver and plasma vitamin E levels when these diets were fed to chicks. Lipids are another class of compounds susceptible to oxidation. Miles et al. (1998) found that replacing poultry diets containing 300 ppm copper sulfate with 300 ppm IntelliBond copper resulted in 17-35% less primary lipid oxidation and 7-45% less secondary lipid oxidation. Enzymes added to the diet are also susceptible to degradation by free metals. Phytase fed to improve phosphorus availability in poultry diets showed 16% greater retention in feed when formulated with IntelliBond copper compared to copper sulfate, indicating a prevention of phytase degradation during feed storage (Liu et al., 2005). Using a less reactive trace mineral source appears to protect vitamins, lipids, and enzymes in feed, ensuring the nutrients formulated retain their optimum quantity and quality.

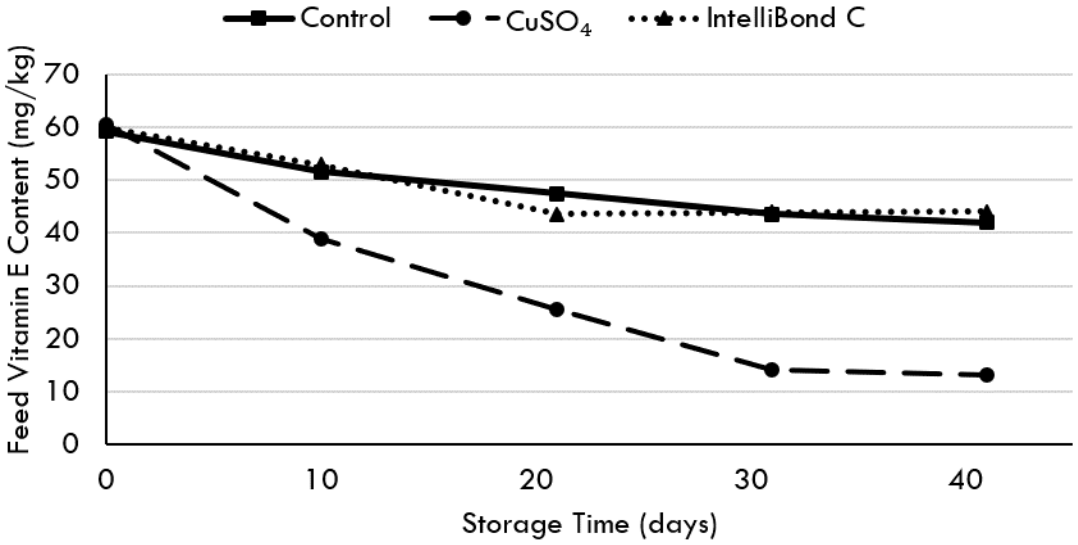


Figure 2. Lu et al., 2010. Feed vitamin E content over time in a poultry diet containing no added copper (Control) or 200 ppm copper from either copper sulfate ($CuSO_4$) or IntelliBond C

Palatability

Studies across a variety of species have shown that, when given the choice, animals prefer to consume IntelliBond rather than sulfate sources of trace minerals. The reason behind increased palatability may be related to the high reactivity of sulfate trace minerals due to their weak ionic bond and release of free metal stimulating an averse metallic taste. In both broilers and layers, as copper sulfate is added to a diet, intake gradually decreases. However, when the copper sulfate is replaced with IntelliBond copper, intake is maintained (Miles et al., 1998; Kim et al., 2016). Similarly, when pigs are presented a choice between feeds formulated with either copper sulfate or IntelliBond copper, a greater proportion of IntelliBond-formulated feed is consumed (Coble et al., 2014). Ruminant studies have also shown an increase in preference of IntelliBond copper, zinc, and manganese over both sulfate and organic sources in a creep-fed pre-weaning supplement, a weaned calf supplement, and a cooked molasses block (Wiebusch et al., 2015; Caramalac et al., 2017; Ranches et al., 2018; Figure 3). To further investigate, Caramalac et al. (2017) conducted four separate trials looking at how each individual metal source (Cu, Zn, or Mn) affected preference. When given the opportunity to select between sulfate, organic, and IntelliBond sources, calves consumed more supplement containing IntelliBond versus organic or sulfate sources of copper (Trial 1), zinc (Trial 2), or manganese (Trial 3). In the fourth trial, all three elements were combined within a single supplement, and the calves had an overwhelming preference for IntelliBond mineral over organic or sulfate sources (82.9, 10.4, and 6.7% of total supplemental intake, respectively). It is remarkable these effects are consistently seen across livestock species, and this partiality may be explained by an evolved aversion to metallic-tasting compounds in their free ionic state.

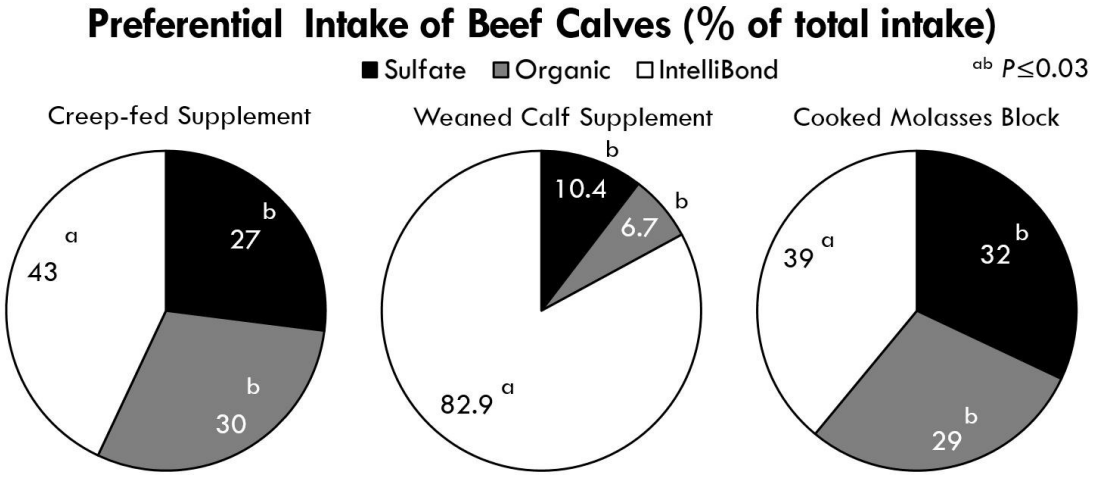


Figure 3. Preferential intake of beef calves as a percentage of total intake when fed a creep-feed mineral (left; Wiebusch et al., 2015), meal supplement (center; Caramalac et al., 2017), or a cooked molasses block (right, Ranches et al., 2018) formulated with minerals from sulfate, organic, or IntelliBond sources of Zn, Cu, and Mn

Bioavailability

One of the goals of trace mineral supplementation is to ensure minerals are available to the animal during different situations that might affect trace mineral bioavailability. Dietary antagonists such as fiber, molybdenum, sulfur, iron, and imbalances in zinc and copper have the potential to affect absorption of different metals (Spears, 2003). Most antagonistic reactions occur in the rumen. If free metals are released in this environment, there is the potential to bind to antagonists, rendering them too tightly bound and causing them to pass through the lower GI tract and past absorption sites in the small intestine. Thus, the reactivity of trace minerals in the rumen is a large determining factor in the potential to bind with antagonists. When dosed in the rumen, sulfate sources of copper, zinc, and manganese were shown to be significantly more soluble than IntelliBond trace minerals (Caldera et al., 2019; Figure 4). Because copper, zinc, and manganese sulfate are bonded to their ligand via ionic bonds, solubility in the rumen is analogous to dissociation, meaning soluble sulfate trace mineral sources release free ionic metals and animals are more prone to antagonist-induced deficiencies. Furthermore, Caldera et al. (2019) investigated the binding strength of these minerals in digesta 12 hours post-bolus using dialysis against chelating agents. The chelating agent released more metal from rumen digesta of IntelliBond- versus sulfate-dosed animals, indicating the marked increase in rumen soluble mineral from sulfates resulted in the metal becoming too tightly bound to antagonistic complexes. IntelliBond begins to solubilize in acidic conditions (Spears et al., 2004) such as the abomasum and gradually throughout the intestinal tract. These data indicate IntelliBond sources avoid antagonistic interactions early in the digestive tract (i.e., the rumen), ensuring trace minerals reach their site of absorption in an available form with as little interference as possible.

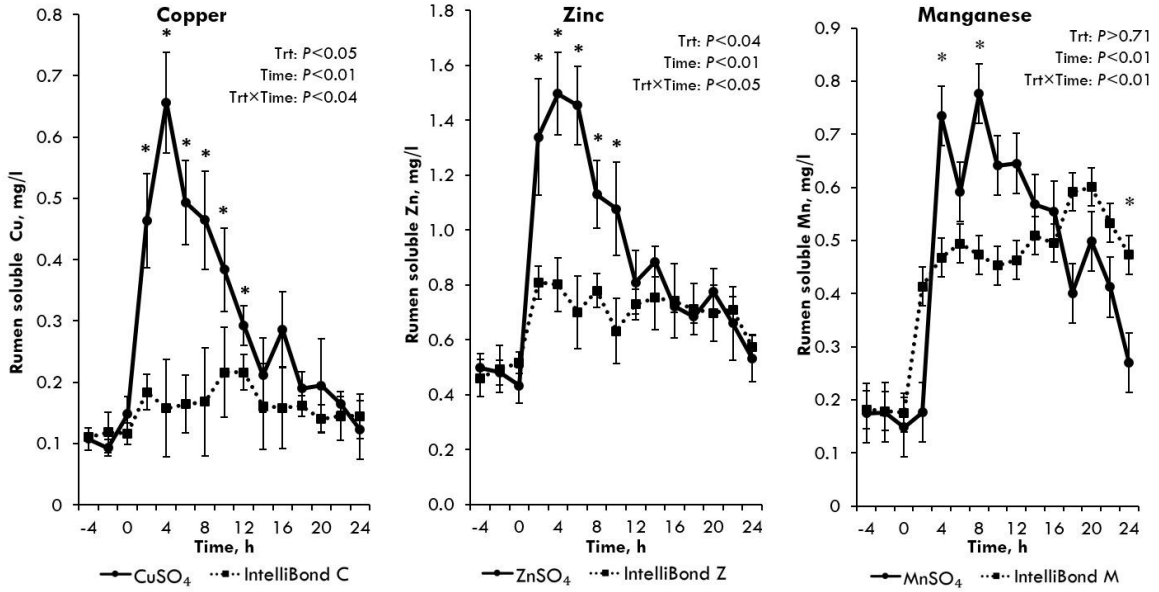


Figure 4. Caldera et al., 2019. Influence of trace mineral source on rumen soluble copper (left), zinc (center), and manganese (right) concentrations

Bioavailability is expressed relative to a standard source, and there are two main approaches: 1) feed mineral at dietary concentrations much higher than the animal's requirement and measure accumulation in tissues, or 2) feed mineral at dietary concentrations below the animal's requirement and measure a specific function. The latter is more practical because it measures the bioavailability of the mineral in a situation when mineral is limiting. However, it is difficult to formulate diets deficient in certain trace minerals using practical feedstuffs (Spears and Hansen, 2008). Therefore, the addition of dietary antagonists are often used to test the ability of mineral sources to overcome the antagonistic challenge. When sulfur and molybdenum (Cu antagonists) were fed to copper-depleted steers, the bioavailability of IntelliBond copper was 1.96x and 1.12x compared to copper sulfate based on liver copper concentrations (Spears et al., 2004; VanValin et al., 2019; Figure 5). Additionally, when steers were depleted and then fed 25 ppm zinc from either zinc sulfate or IntelliBond zinc, the bioavailability of IntelliBond zinc was 2.04x compared to zinc sulfate based on retained zinc as measured via total fecal and urine collection (Shaeffer et al., 2017; Figure 5).

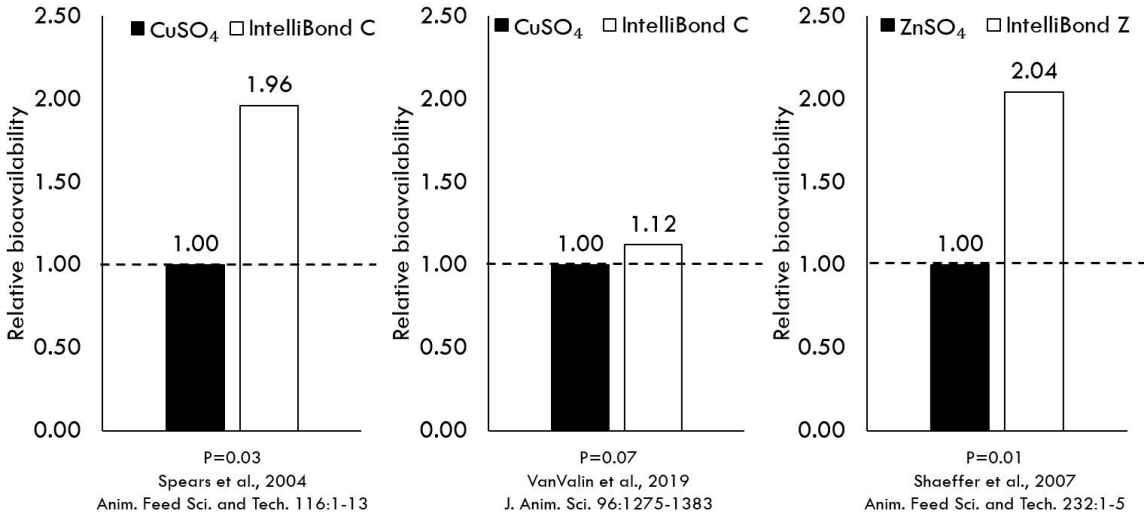


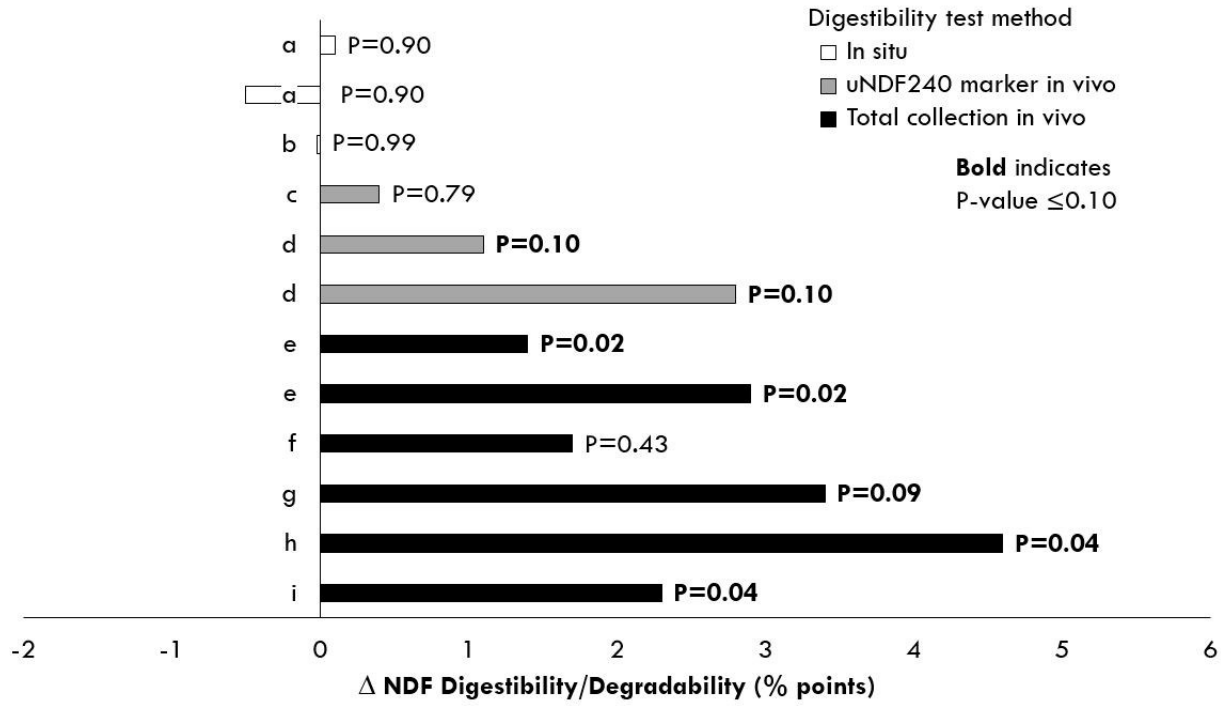
Figure 5. Bioavailability of IntelliBond C during a Mo and S antagonist challenge (left and center; Spears et al., 2004; VanValin et al., 2019) or IntelliBond Z following a Zn depletion period (right; Shaeffer et al., 2017) expressed relative to respective sulfate sources

Digestibility

It is well understood that microbial activity in the rumen is essential to promote optimized neutral detergent fiber digestibility (NDFd), which is fundamental to the production of volatile fatty acids as an important energy source for the ruminant. Metals such as copper, silver, and zinc have known antimicrobial properties that are utilized as antimicrobial agents and antibiotic alternatives in other industries (Lemire et al., 2013). Therefore, reactive trace mineral sources are counterproductive because, once broken down in the rumen, the metal ion (zinc or copper) originally linked within the sulfate ligand now possesses these antimicrobial properties. Consequently, these free metal ions can potentially harm the beneficial fibrolytic bacteria. Research investigating how trace

minerals may affect microbial function dates back to the 1950s. Several in vitro studies have indicated even very low amounts of added copper are highly toxic to cellulolytic bacteria (Sala, 1957; Hubbert et al., 1958; Martinez and Church, 1970; Ward and Spears, 1993). There is a recognized rumen microorganism requirement for zinc (Durand and Kawashima, 1980); however, the rumen microbe requirement for zinc appears to be quite low. Two separate studies conducted using washed suspensions of rumen microorganisms (therefore eliminating any soluble zinc from the basal diet) found either no change or an increase in cellulose digestion with low levels of added zinc (Hubbert et al., 1958; Martinez and Church, 1970). However, the rumen zinc requirement is likely quite small and readily met by zinc within the basal diet, as dry matter digestibility was similar in lambs and calves fed a zinc-deficient diet compared to those supplemented with adequate amounts of zinc (Miller et al., 1966; Somers and Underwood, 1969). Additionally, both washed rumen microbe studies found that continuing to add increasing levels of zinc inhibited cellulose digestion in these suspensions. A more recent in vitro study observed reduced cellulose digestion and cellulolytic bacteria concentration after 24 hours of incubation with 50 µg/mL zinc (Eryavug and Dehority, 2009). Therefore, the zinc requirements of rumen microorganisms appear to be very low relative to zinc requirements of the animal.

To further evaluate the impact of trace mineral source on a more practical level, researchers from multiple universities have looked at how level or source of trace mineral impact digestibility in vivo. Lambs supplemented 100 ppm copper for 30 days had 7.7 points lower dry matter digestibility compared to those receiving 5 ppm copper (Goodrich and Tillman, 1966). Additionally, digestible dry matter intake tended to decrease with increasing ruminal doses of zinc in heifers (Arelovich et al., 2000). In terms of source, supplementing a covalently bonded trace mineral source can prevent the negative digestibility effects of reactive trace minerals by minimizing the amount of free ionic metal in the rumen. In lambs supplemented with organic trace minerals, increased NDFd and ADF digestibility have been observed (Garg et al., 2008; Hassan et al., 2011). In a wide range of studies comparing how sulfate trace minerals affect NDFd relative to IntelliBond trace minerals, research has found an improvement in NDFd ranging from 1.1 to 4.6 points with IntelliBond trace minerals relative to sulfate sources (Figure 6). As Oba and Allen (1999) suggest, a one-point change in NDFd can translate to a 0.17 kg increase in dry matter intake and a 0.25 kg increase in 4% fat-corrected milk. These studies were conducted under practical trace mineral feeding levels (5-25 ppm Cu [average 12 ppm Cu], 30-120 ppm Zn [average 56 ppm Zn], and 15-60 ppm Mn [average 36 ppm Mn]) indicating that a component as seemingly insignificant as trace mineral source can have a profound impact on digestibility and energy status in today's high producing ruminants.



a=Genther and Hansen. 2015. J. Dairy Sci. 98:566-573
b=Micronutrients Trial #2017D103CACZM
c=Micronutrients Trial #2017D123USCZM
d=Miller et al., 2019. J. Dairy Sci. (ADSA Abstract #338)
e=Faulkner and Weiss, 2018. J. Dairy Sci. 100:5358-5367
f=VanValin et al., 2018. J Anim. Sci. 96:5336-5344
g=Caldera et al., 2019. J. Anim. Sci. 97:1852-1864
h=Micronutrients Trial #2017R120USCZM
i=Guimaraes et al., 2019. J. Anim. Sci. (ASAS Abstract #414)

Figure 6. Change in neutral detergent fiber (NDF) digestibility relative to sulfate sources of trace minerals in various studies.

Summary

The primary goals of trace mineral supplementation are to meet the nutritional demands of today's high producing animals and to provide a bioavailable mineral source that does its job without negatively interacting with other components of the diet. Providing a high-quality improved trace mineral source as a replacement for sulfate trace minerals ensures proper trace mineral nutrition without the negative side-interactions sulfate minerals may have on diet stability, palatability, bioavailability, and digestibility that may do more harm than good.

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Current Concepts in Fiber Digestion: Focus on Source of Forage and Trace Mineral

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Introduction

Corn silage is a common source of digestible fiber for lactating dairy cows. In New York State in 2018, 445,000 acres of corn silage were harvested to produce 8,455,000 tons (USDA/NASS, 2019). There are differences between hybrids in the quantity and quality of digestible fiber provided. Corn silage having the brown midrib gene mutation, either BM1 or BM3, had increased neutral detergent fiber digestibility (NDFd) and lower lignin content compared to conventional corn silage (Oba and Allen, 1999a; Hassanat et al., 2017).

Historically, fiber has been measured as crude fiber or NDF, but in the last decade, new analyses have allowed for better fractionation of NDF (Raffrenato et al., 2018). The new analyses are an in vitro fermentation at various time points (30, 120, and 240 h) and are called undigested NDF (uNDF; Raffrenato et al., 2018). The uNDF at 240 h is a measure of the indigestible NDF (iNDF) and allows for an accurate estimation of potentially digestible NDF (pdNDF) which is the fiber that the cow has the opportunity to digest (Cotanch et al., 2014).

Recently we conducted a corn silage hybrid study over three years (2015 - 2017) comparing BM1, BM3, and non-BMR hybrids in quantity and quality (Miller et al., 2018). Using the newly defined fiber fractions, we found that BM3 hybrids had higher NDFd, lower uNDF at 240 h, and higher pdNDF (% of NDF) than BM1 and non-BMR hybrids (Miller et al., 2018). Oba and Allen (2000a) reported that cows fed BM3 corn silage-based diets had higher dry matter intake (DMI) and milk production compared to cows fed CON corn silage-based diets. They also reported faster passage rates of NDF and iNDF in cows fed BM3 corn silage-based diets compared to CON corn silage-based diets (Oba and Allen, 2000a, 2000b, and 2000c).

Some trace minerals are required in small amounts for proper rumen microbe function; however, requirements of rumen microorganisms appear to be very low relative to requirements of the animal. The source of trace mineral affects the solubility in the rumen and could have negative effects on cellulolytic bacteria or bind to undigested fractions (e.g., fiber fractions) that pass from the rumen (Torre et al., 1991; Genther and Hansen, 2015; Faulkner et al., 2017). A recent study showed that feeding hydroxy Cu, Mn, and Zn minerals increased total tract NDF digestibility compared to sulfate sources in forage- and by-product-based dairy cattle diets (Faulkner and Weiss, 2017). A greater difference in total tract NDF digestibility in the forage-based diets between the sulfate

(STM) and hydroxy trace minerals (HTM) suggests that the source of NDF potentially influences the effect of source of trace mineral on NDF digestibility.

Based on those findings, we investigated the effect of source of corn silage and trace mineral on lactation performance and fiber digestibility. We expected that the increased fiber digestibility of BM3 corn silage would allow for higher DMI and milk production, and the decreased solubility in the rumen of HTM in the rumen would allow for higher total tract digestibility of NDF.

Miner Institute Study: Source of Corn Silage and Trace Mineral

Dietary Treatments

To test the effect of source of corn silage and trace mineral, we conducted a study in 2018 using four diets with either conventional or brown midrib-3 corn silage and either a sulfate source of Cu, Zn, and Mn or hydroxy trace minerals (IntelliBond Cu, Zn, and Mn; Micronutrients LLC USA, Indianapolis, IN). The diets contained approximately 54.6% corn silage, 2.3% chopped wheat straw, 43.1% concentrate mix, and either 0.033% STM or 0.022% HTM (Table 1). The objective for the substitution of the corn silage on a 1:1 DM basis was to allow differences in fiber fractions of the diet to be determined by the source of corn silage and their respective fiber content and digestibility.

Table 1. Ingredient composition (% of DM) of diets containing either CON corn silage or BM3 corn silage with either STM or HTM fed to lactating Holstein cows.

	Diets			
	CON		BM3	
Item	STM	HTM	STM	HTM
Conventional corn silage (CON)	54.6	54.6	.	.
Brown mid-rib corn silage (BM3)	.	.	54.6	54.6
Straw	2.3	2.3	2.3	2.3
Concentrate mix				
Other	43.1	43.1	43.1	43.1
Premix				
Cu sulfate	0.004	.	0.004	.
Mn sulfate	0.012	.	0.012	.
Zn sulfate	0.017	.	0.017	.
Hydroxy Cu	.	0.002	.	0.002
Hydroxy Mn	.	0.011	.	0.011
Hydroxy Zn	.	0.009	.	0.009

The amylase-modified NDF on an organic matter basis (aNDFom) content of the CON diets was higher than BM3 diets due to differences in corn silage (36.2 vs. 32.1% of DM; Table 2). The BM3 diets had higher NDFd 30-h and lower uNDF at 240 h than the CON diets (62 vs. 55.7% of aNDFom; 6.9 vs. 8.6% of DM, respectively). The starch content of the CON diets was lower than BM3 diets, and this was due to a decrease in the starch content of the CON corn silage during the study (21.9 vs. 26.2% of DM). The trace mineral concentrations were similar across diets and were chosen to be similar to on-farm diets typically formulated for high-producing dairy cows.

Table 2. Calculated diet composition based on chemical analysis of ingredients fed to lactating Holstein cows.

	Diets			
	CON		BM3	
Item	STM	HTM	STM	HTM
CP, % of DM	15.0	15.3	15.6	15.4
aNDFom, % of DM	36.3	36.0	32.1	32.0
30-h aNDF digestibility, % of aNDFom	56.0	55.3	61.9	62.0
Undigested NDFom at 240-h, % of DM	8.6	8.6	6.9	6.9
Potentially digestible NDF, % of aNDFom	76.3	76.1	78.4	78.2
Starch, % of DM	21.8	21.9	26.2	26.1
Copper, mg/kg of DM	18	17	17	16
Manganese, mg/kg of DM	61	68	60	64
Zinc, mg/kg of DM	101	93	107	88

When feeding these dietary treatments (Table 1 and 2), we expected the cows fed the BM3 diets to have higher DMI and milk production (Oba and Allen, 2000a; Hassanat et al., 2017) and trace minerals to have minimal effect on DMI and milk production (Faulkner and Weiss, 2017). There was a corn silage effect on DMI as the cows fed the BM3 diets had greater DMI than cows fed the CON diets (28.1 vs. 27.5 kg/d; Table 3). The increased fiber digestibility and lower uNDF240 of the BM3 corn silage allowed the cows to have greater intake. There was also a trace mineral effect on DMI as the cows fed the HTM diet had greater DMI than cows fed the STM diets. In contrast to our results, a similarly designed study reported no effect of trace mineral source on DMI (Faulkner and Weiss, 2017).

Cows fed the BM3 diets had higher milk and energy-corrected milk (ECM) yield compared to the cows fed the CON diets (47.0 vs. 44.7 kg/d; 47.6 vs. 46.2 kg/d, respectively). With the greater dietary starch content, lower NDF content, and feed intake of the cows fed the BM3 diets; it is not surprising that they produced more milk than the cows fed the CON diets. There was a corn silage effect on feed efficiency when expressed as milk per DMI, but there was no corn silage effect when feed efficiency was expressed as ECM per DMI.

Table 3. Least squares means of DMI and milk production data from lactating Holstein cows fed diets containing either CON corn silage or BM3 corn silage with either STM or HTM.

	Diets							
	CON		BM3			*P*-value		
Item	STM	HTM	STM	HTM	SE	CS	TM	CS x TM
DMI, kg/d	27.4	27.6	27.5	28.6	0.6	0.02	0.01	0.11
DMI, % of BW/d	4.12	4.16	4.18	4.30	0.1	<0.01	0.01	0.26
Milk, kg/d	44.8	44.6	46.2	47.7	1.1	<0.01	0.21	0.12
ECM, kg/d	46.4	45.9	46.9	48.2	1.2	0.02	0.47	0.17
Milk/DMI, kg/kg	1.63	1.62	1.68	1.67	0.05	<0.01	0.22	0.80
ECM/DMI, kg/kg	1.69	1.66	1.70	1.69	0.03	0.43	0.21	0.76

When we assessed total tract digestibility (TTD), the cows fed the BM3 diets had higher TTD of organic matter (OM) and lower TTD of starch compared to the cows fed the CON diets (74.1 vs. 72.3%; 95.1 vs. 99.1%, respectively; Table 4). The BM3 corn silage has a higher amount of potentially digestible NDF than CON corn silage which could mean that more of the fiber might be digested in the cow (Table 2). However, in similar studies (Hassanat et al., 2017; Oba and Allen, 2000c), they reported no differences in TTD of OM in cows fed either BM3 or CON corn silage. Ferraretto and Shaver (2015) reported lower total tract starch digestibility for cows fed the BM3 diets compared to cows fed CON diets due to greater kernel vitreousness of BM3 corn silage hybrids.

The cows fed the HTM diets had a tendency for higher TTD of aNDFom than the cows fed the STM diets (56.9 vs. 54.9%; Table 4). This was in agreement with Faulkner and Weiss (2017) who reported cows fed diets with HTM had a higher TTD of NDF compared to cows fed diets with STM. Oba and Allen (1999b) reported that a one-unit increase in vivo total tract NDF digestibility of TMR was associated with a 0.42-kg increase in DMI. The 2.8% difference in total tract digestibility of aNDFom between the cows fed the BM3-HTM and BM3-STM diets would equate to a 1.18-kg increase in DMI, which would explain the 1.1-kg difference observed in DMI.

Table 4. Least squares means of total tract digestibility data from lactating Holstein cows fed diets containing either CON corn silage or BM3 corn silage with either STM or HTM.

	Diets							
	CON		BM3			*P*-value		
Item	STM	HTM	STM	HTM	SE	CS	TM	CS x TM
OM, % of OM	72.1	72.5	74.2	74.0	0.7	0.01	0.88	0.71
Starch, % of starch	99.0	99.1	95.0	95.2	0.2	<0.01	0.48	0.90
aNDFom, % of aNDFom	54.4	55.5	55.4	58.2	1.2	0.12	0.10	0.52
pdNDF, % of pdNDF	70.7	71.7	70.4	72.7	1.4	0.80	0.20	0.65

Summary And Perspectives

Minerals Matter

This study evaluated the effect of source of corn silage and trace mineral on fiber digestibility and lactation performance. Cows fed the BM3 diets had higher DMI and ECM than cows fed the CON diets. Source of trace minerals had an effect on DMI with the cows fed the HTM diets having higher DMI than the cows fed the STM diets. This DMI difference between the HTM and STM diets can be accounted for by the difference in total tract digestibility of aNDFom. The effect was greater for the cows fed the BM3 diets compared to the cows fed the CON diets. Source of trace minerals influences DMI and total tract digestibility of aNDFom and should be taken into consideration when formulating diets for high-producing dairy cows.

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Effects of Low Feed Intake on Gastrointestinal Function[1]

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Introduction

Ration formulation and evaluation systems have advanced significantly over the past 15 yr and how provide a more accurate and detailed prediction for the performance of beef and dairy cattle. Using these models, it is clear that to promote milk and milk component yield for dairy cattle and gain:feed (**G:F**), highly fermentable diets are needed. Feeding highly fermentable diets increases short-chain fatty acid (**SCFA**) production in the rumen and microbial protein over diets with a lower fermentability. Thus, this strategy results in a greater supply of metabolizable energy and increases metabolizable protein supply. On the other hand, feeding rapidly fermentable diets can result in excessive fermentation rates that lead to low ruminal pH, high rumen osmolality, and can increase the concentration of antigenic compounds within the rumen. Obviously, there is a fine balance between diets that promote rapid fermentation and those that cause deleterious effects.

As feeding strategies for ruminants are implemented to promote SCFA production and metabolizable protein, understanding factors that alter the ability of the gastrointestinal tract to maintain its critical functions (**GIT**) is essential. Historically, the role of the GIT was thought to be limited to facilitating digestion, nutrient absorption, and the transit of feed through the GIT. That said, it has been recognized that the GIT plays a critical role in regard to preventing or limiting the passage of non-desired molecules (e.g. microbial associated molecular patterns, pathogenic microbes, and other byproducts arising form fermentation [histamine]) from entering portal circulation. This process is referred to as selective permeability and is one critical component of GIT barrier function. Failure to prevent these molecules from entering portal circulation would result in a systemic immune response. In addition to absorptive and barrier function processes, the gastrointestinal tract also serves as a communicative organ. Recent studies have demonstrated that the GIT can detect nutrients in the lumen that leads to an up regulation of absorptive processes. Moreover, endocrine and paracrine secretions can facilitate communication with the rest of the body including the nervous system. The GIT also communicates with its microbial inhabitants. While much of this research has been conducted in monogastrics, Weimer et al. (2010) has reported that ruminants may also regulate the microbial community structure of the rumen. In that study, they surveyed ruminally cannulated dairy cattle fed the same diet, with similar days in milk, and housed in the same facility. They selected 2 cattle that had the greatest differences in ruminal pH, ruminal SCFA concentrations, and microbial community structure. Subsequently, they completely evacuated the ruminal contents and refilled the rumen with the other cows' contents. Over the next 65 d, they evaluated the microbial community structure and found

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that the microbial profile returned to a profile that resembled the native community. This study provided new evidence that cattle have the ability to at least partially regulate the microbial community structure.

Relevance of Low Feed Intake in Commercial Settings

Beef and dairy cattle are often, albeit inadvertently, exposed to periods of low feed intake (**LFI**). An example for a transient period of low feed intake is for dairy calves during the weaning transition where the increase in starter intake may not compensate for the reduction in milk or milk replacer DMI (Wood et al., 2015). Beef calves also likely reduce DMI in association with weaning as calf walking activity and mealtime is markedly reduced (Price et al, 2003; Haley et al., 2005) although weaning methods can have a significant impact on the negative behavioural response (Haley et al., 2005; Wiese et al., 2015). However, no studies have measured calf DMI prior and post weaning, and only 1 study has attempted to measure calf DMI post-weaning and reported no differences among weaning methods (Wiese et al., 2015). Nevertheless, newly received calves certainly experience extended periods of low feed intake. For example, Hutcheson and Cole (1986) reported that calves in the first, second, and third week after arrival at a feedlot often only consume DM at a rate of 0.5 to 1.5, 1.5 to 2.5%, and 2.5 to 3.5% of BW, respectively. Thus, newly received cattle experience an extended duration of low feed intake and variable extents for the magnitude of intake depression (Hutcheson and Cole, 1986; Loerch and Fluharty, 1999). The LFI occurring for newly received cattle is likely related to the complete feed and water deprivation that occurs during transportation (González et al., 2012) and as part of the response to the change in social structure, physical housing methods, and diets that occur upon arrival.

Transition dairy cattle also experience a transient period of LFI around calving. In fact, a literature review reported that the severity of FR ranges from a reduction of intake up to 68% on d 1 pre-partum relative to 21 d pre-partum for dairy cattle (Hayirli et al., 2002). Although the range in low feed intake is large, on average, cattle reduce their feed intake by 33% with nearly 90% of that reduction occurring in the last week prior to calving. More recent studies have supported the results of Hayirli et al. (2003) as Janovick and Drackley (2010) demonstrated that transition dairy cattle reduced intake by up to 30% as parturition approached and Penner et al., (2007) reported greater than a 30% reduction in primiparous heifers. The extent of LFI can be exacerbated for transition cows in association with infectious diseases or metabolic and digestive disorders (e.g. displaced abomasum, ketosis; Van Winden et al., 2003; Goldhawk et al., 2009) and numerous studies have been able to identify associations between risk for metabolic disease and low feed intake pre-partum (Huzzy et al., 2009). Thus, it is clear that dairy cattle experience a period of low feed intake prior to calving and this is coupled with a rapid increase in DMI and diet fermentability following calving.

Environmental conditions can also induce LFI. Heat stress has been reported to lead to marked reductions in DMI (Maust et al., 1972; Knapp and Grummer, 1991; Holter et al., 1996). The magnitude of reduction in DMI differs based on the severity of the heat stress but under experimental conditions, Baumgard et al. (2011) reported a reduction in

DMI of 18% when exposed to a temperature-humidity index of 64 and Wheelock et al. (2010) reported a DMI reduction of 30% when exposed to the same temperature-humiditiy index as Baumgard et al. (2011). It appears that the magnitude of DMI depression varies among cattle when exposed to the same severity of heat stress and it is likely that the depression in intake is exacerbated with greater severity of the thermal heat load.

Effects of Low Feed Intake on Function of the Gastrointestinal Tract

Although LFI may be transient, past studies in sheep have demonstrated that periods of complete feed deprivation can have negative consequences on the absorptive and barrier functions of the rumen epithelium. For example, the transport of Na^+, Cl^-, Mg^{2+}, and SCFA were reduced by approximately 50% for sheep exposed to a 48-h period of feed deprivation (Gäbel et al., 1993). With respect to barrier function, Gäbel and Aschenbach (2002) demonstrated that the passive passage of a small hydrophilic molecule (3-O-methyl-*α*-D-glucose) was increased following feed deprivation in sheep. While the previous studies provided evidence to suggest that LFI compromises GIT absorptive and barrier function, the experimental model evaluated 48 h of complete feed deprivation; a condition that is not common under industry practices. To address this limitation, we initiated a series of experiments to assess what severities of LFI induce changes in the function of the GIT and to evaluate how the GIT recovers in response to LFI.

In the first study (Zhang et al., 2013a), we assessed the effect of differing severities of LFI by restricting cattle to 75, 50, or 25% of their ad libitum DMI for a 5-d duration. Following exposure to LFI, cattle were provided feed, offered as a total mixed ration, ad libitum and monitored for 3 consecutive weeks. The diet (30% barley silage, 30% grass hay, 32% rolled barley grain, and 8% of a pelleted barley supplement containing minerals and vitamins) was common among all cattle and periods such that we only evaluate the effect of LFI. Our results showed that in response to LFI, the concentration of SCFA in the rumen decreased in a dose-dependent fashion as heifers restricted to 75% had concentrations that were less than when fed ad libitum, concentration of SCFA for heifers restricted to 50% were less than 75%, and those restricted to 25% had concentrations less than those restricted to 50% of their ad libitum DMI. The reduction in SCFA concentrations is logical as DMI decreased, but warrants mention as SCFA provide the bulk majority of the metabolizable energy supply for ruminants and provision of fermentable carbohydrate stimulates the metabolizable protein supply. Corresponding to the reduction in DMI, there was a dose-dependent increase in mean ruminal pH with greatest increases occurring with the most severe LFI. The consequence of the LFI resulted in a tendency for reduced SCFA absorption across the reticulo-rumen and the rate of absorption tended to be reduced as the severity of the LFI increased. Moreover, permeability of the GIT was increased for heifers exposed to 25% of their ad libitum intake. A second study (Albornoz et al., 2013a,b) also found that exposure to LFI (25% of ad libitum DMI) reduced SCFA concentration in the rumen, increased ruminal pH, and reduced SCFA absorption across the reticulo-rumen. It should be recognized that not only did exposure to LFI reduce dietary nutrient supply, but capacity for nutrient absorption was also compromised and permeability was increased. This suggests that exposure to

low feed intake, regardless of the cause (i.e. weaning, heat stress, parturition, transportation, etc.), is likely to reduce metabolizable energy and protein absorption and may predispose cattle to systemic inflammation.

While our research demonstrated that LFI reduces aspects of GIT function, there was no indication for the recovery time required. To address this limitation Zhang et al., (2013b) evaluated the recovery response upon return to full feed allocation. A particularly interesting finding was that the severity of LFI alone caused variability in how rapid heifers returned to ad libitum DMI (Figure 1). For example, heifers exposed to LFI at 25% of their voluntary intake required 3 wk to return to pre-LFI DMI, while those restricted to 75% of their voluntary intake only required 1 wk. This suggests that in the absence of other challenges (parturition, weaning, heat stress, etc.), the severity of LFI can affect the recovery rate for DMI.

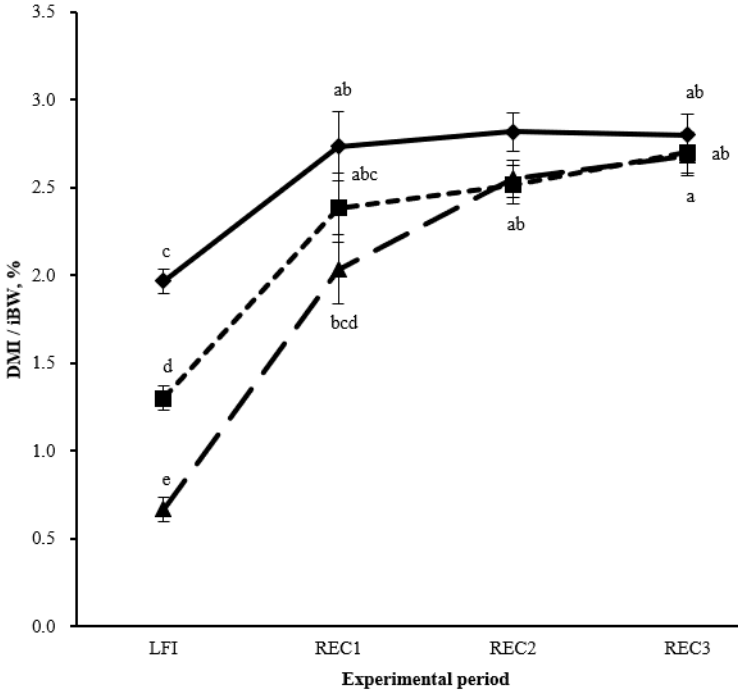

Figure 1. Voluntary recovery of dry matter intake during a 3-wk recovery period (REC1 = 1st week of recovery, REC2 = 2nd week of recovery, and REC3 = 3rd week of recovery) after a 5-d exposure to low feed intake (LFI). For the LFI, heifers were exposed to 75% (solid line with diamonds), 50% (short-dashed line with squares), or 25% (long dashed line with triangles) of their voluntary intake. The DMI was 2.7% of BW prior to LFI. There was a treatment × period interaction ($P < 0.001$) and letters indicate means that differ ($P < 0.05$). Adapted from Zhang et al., (2013b).

Another interesting finding was that despite heifers being fed a diet consisting of 60% forage, return to ad libitum intake resulted in induction of ruminal acidosis and this response was dose-dependent. Thus, heifers exposed to the greatest severity of LFI (25% of ad libitum intake) had the lowest mean ruminal pH (5.88) in the first week of recovery and those restricted to 75% had the greatest mean ruminal pH; although the mean was still only 6.16. On average, the duration that pH was less than 5.5 was 6 h/d during the first week of recovery indicating substantial exposure to low pH conditions. The severity of LFI also affected SCFA absorption during recovery with heifers restricted to 50 and 25% requiring more time to recovery than those restricted to 75%, and permeability of the GIT did not recover for heifers restricted to 25% of their voluntary intake. This data suggests that cattle may require up to 3 wk for the GIT function to recovery after exposure to low feed intake depending on the severity of the LFI event. Given that a greater severity of LFI required more time for recovery, transition dairy cattle that experience metabolic or infectious diseases or feedlot cattle experiencing infectious disease will likely be affected to a greater extent. Moreover, as cattle start to return to their ad libitum intake, they are susceptible to ruminal acidosis, even with low initial feed intake and diets with a substantial forage content.

The data above describe effects of low feed intake on GIT function under artificial conditions and industry relevance could be questioned. To address this issue, we evaluated the changes in DMI and total tract permeability of the GIT for dairy calves at weaning (Wood et al., 2015). In this study, 14 Holstein bull calves were used and fed milk replacer at a rate of 15% of their BW (adjusted weekly). The milk replacer was included at 150 g (DM basis)/1 L of water. Calves were exposed to a 42-d milk feeding protocol followed by a 7-d step down weaning program (**WEAN**) or were not weaned (**CON**). The step-down weaning protocol was effective at increasing starter intake as WEAN calves had greater intake than the CON. Calves in the WEAN treatment experienced did not increase BW to the same extend during the step-down period further indicating a reduction in nutrient intake associated with weaning. To evaluate GIT permeability, we orally dosed Cr-EDTA and measured its appearance in urine. This approach is the same method used and validated by Zhang et al. (2013). We observed that permeability of the GIT decreased from wk 2 of age to wk 4 of age and for the CON continued to decrease to wk 6. However, calves that were weaned followed the same trend until wk 6 where weaning caused a marked increase in permeability of the GIT. This supported the work of Zhang et al. (2013a,b) showing that reductions in DMI and nutrient intake are associated with increased GIT permeability.

Strategies to Minimize the Negative Effect of Low Feed Intake and Accelerate Recovery of the Gastrointestinal Tract

Based on the data above, it appears that LFI consistently decreases aspects of GIT function. However, this data does not indicate what management strategies can be used to mitigate the negative effect caused by LFI or accelerate the recovery response. It should be recognized that there are instances where LFI could be predicted (i.e. weaning, transportation) and hence dietary scenarios could be put in place to minimize the negative effect of LFI. Alternatively, there are many scenarios where LFI cannot be

accurately predicted (metabolic disorders and infectious disease) or the timeline may be difficult to accurately predict (parturition and heat stress). Thus, there is a need to evaluate strategies that can either mitigate the effect of LFI when it is predictable and, more practically, accelerate the recovery following a period of LFI.

For predictable exposure to LFI, we evaluate whether altering the forage:concentrate ratio of the diet could be a mechanism to mitigate the negative effect (Albornoz et al., 2013a,b). In this study heifers were either fed a high forage diet (92% forage with 50% of the forage supplied from barley silage and 50% from grass hay, and 8% of a pelleted barley supplement containing minerals and vitamins; DM basis) or a moderate forage diet (30% barley silage, 30% grass hay, 32% rolled barley grain, and 8% of a pelleted barley supplement containing minerals and vitamins; DM basis). These treatments were used as feeding a high-forage diet may increase ruminal retention thus decreasing the severity of the LFI event, while feeding a moderate forage diet provides a greater nutrient supply per unit of feed intake. Heifers were exposed to a 5-d period of LFI with feed restricted to 25% of voluntary intake. As previously reported LFI reduced SCFA in the rumen, increased mean pH, and reduced the rate of SCFA absorption. Feeding the high forage diet prior to and during LFI, increased serum non-esterified fatty acids (an indicator of adipose tissue mobilization) to a greater extent than the moderate forage diet, and reduced the rate of recovery for DMI after return to voluntary intake. Heifers fed the high forage diet prior to and during the LFI also had greater risk for ruminal acidosis during the recovery period. Surprisingly, the diet fed prior to and during LFI did not affect the rate of recovery for SCFA absorption. This indicates that diets with a high forage content may have a more deleterious effect than diets with a moderate forage content when fed during a period of LFI, likely because of reduced nutrient density.

On the other hand, most episodes of LFI will not be accurately predicted but can be detected with adequate feed bunk management and behavioural responses. Given that cattle are susceptible to ruminal acidosis during recovery after LFI, we evaluated the effect of feeding a moderate or high forage diet during recovery. Interestingly, heifers fed the high forage diet following LFI resumed voluntary DMI within the first wk of recovery whereas; heifers fed the moderate forage diet required 3 wk. The response of the moderate forage-fed heifers mirrored that reported by Zhang et al. (2013b). Feeding the high-forage diet during recovery also allowed heifers to stabilize mean pH and those fed the high forage diet did not experience ruminal acidosis. Heifers fed the moderate forage diet had mean pH below 6 during the first week of recover whereas, heifers fed the high forage diet had a mean pH above 6.3. The induction of ruminal acidosis during the first week of recovery was also evident as heifers fed the moderate forage diet spent over 4 h below pH 55 while those on the high forage diet spent less than 20 min below the same threshold. Although ruminal fermentation parameters were affected, there were no differences in the rate of SCFA absorption between recovery treatment strategies.

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

Summary

Low feed intake negatively affects absorptive and barrier functions of the gastrointestinal tract and predisposes cattle to ruminal acidosis. Feeding a diet with a greater energy density prior to low feed intake can help accelerate the recovery response as can feeding a diet with a high forage content after a period of low feed intake. In a finishing scenario, this can be accomplished simply by increasing the proportion of forage in the diet (by approximately 11% units). We also report that feeding additives that support gastrointestinal function (e.g. betaine, superoxide dismutase, and butyrate), can help accelerate the recovery response for the gastrointestinal tract. These results demonstrate

an industry-relevant nutritional challenge along with strategies that can be implemented to mitigate the impact of low feed intake.

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Causes and Metabolic Consequences of Leaky Gut

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Introduction

Suboptimal milk yield limits the U.S. dairy industry's productive competitiveness, marginalizes efforts to reduce inputs into food production, and increases animal agriculture's carbon footprint. There are a variety of circumstances in a cow's life which result in hindered productivity including heat stress, ketosis, rumen and hindgut acidosis, feed restriction, and psychological stress associated with normal animal practices (i.e., pen changes, weaning, shipping). Although these insults have different origins, a commonality among them is increased production of inflammatory biomarkers and markedly altered nutrient partitioning. We and others have generated convincing data strongly implicating intestinally derived lipopolysaccharide (LPS) as sometimes being the culprit in these situations.

Heat Stress

During heat stress (HS), blood flow is diverted from the viscera to the periphery in an attempt to dissipate heat, and this leads to intestinal hypoxia (Hall et al., 1999). Enterocytes are particularly sensitive to hypoxia and nutrient restriction (Rollwagen et al., 2006), resulting in ATP depletion and increased oxidative and nitrosative stress (Hall et al., 2001). This contributes to tight junction dysfunction and gross morphological changes that ultimately reduce intestinal barrier function (Lambert et al., 2002; Pearce et al., 2013), resulting in increased passage of luminal content into portal and systemic blood (Hall et al., 2001; Pearce et al., 2013). Endotoxin, otherwise referred to as LPS, is a glycolipid embedded in the outer membrane of Gram-negative bacteria, which is abundant and prolific in luminal content, and is a well-characterized potent immune stimulator in multiple species (Berczi et al., 1966; Giri et al., 1990; Tough et al., 1997). Immune system activation occurs when LPS binding protein (LBP) initially binds LPS and together with CD14 and TLR4 delivers LPS for removal and detoxification, thus LBP is frequently used as a biomarker for LPS infiltration (Ceciliani et al., 2012). For a detailed description of how livestock and other species detoxify LPS see our recent review (Mani et al., 2012). Endotoxin infiltration into the bloodstream during HS, which was first observed by Graber et al. (1971), is common among heat stroke patients (Leon, 2007) and is thought to play a central role in heat stroke pathophysiology as survival increases when intestinal bacterial load is reduced or when plasma LPS is neutralized (Bynum et al., 1979; Gathiram et al., 1987). It is remarkable how animals suffering from heat stroke or severe endotoxemia share many physiological and metabolic similarities to HS, such as an increase in circulating insulin (Lim et al., 2007). Intramammary LPS infusion increased (~2 fold) circulating insulin in lactating cows (Waldron et al., 2006). In addition, we

intravenously infused LPS into growing calves and pigs and demonstrated >10 fold increase in circulating insulin (Rhoads et al., 2009; Kvidera et al., 2016, 2017c). Interestingly, increased insulin occurs prior to increased inflammation and the temporal pattern agrees with our previous *in vivo* data and a recent *in vitro* report (Bhat et al., 2014) suggesting LPS stimulates insulin secretion, either directly or via GLP-1 (Kahles et al., 2014). The possibility that LPS increases insulin secretion likely explains the hyperinsulinemia we have repeatedly reported in a variety of HS agriculture models (Baumgard and Rhoads, 2013). Again, the increase in insulin during both HS and immunoactivation is energetically difficult to explain as feed intake is severely depressed in both experiments.

Ketosis and the Transition Period

Recently, the concept that LPS impacts normal nutrient partitioning and potentially contributes to metabolic maladaptation to lactation has started to receive attention. Although LPS itself has not been the primary causative focus, general inflammation has been the topic of investigations. Increased inflammatory markers following parturition have been reported in cows (Ametaj et al., 2005; Bionaz et al., 2007; Bertoni et al., 2008; Humblet et al., 2006; Mullins et al., 2012). Presumably, the inflammatory state following calving disrupts normal nutrient partitioning and is detrimental to productivity (Bertoni et al., 2008), and this assumption was recently reinforced when TNFα infusion decreased productivity (albeit without overt changes in metabolism; Yuan et al., 2013; Martel et al., 2014). Additionally, in late-lactation cows, injecting TNFα increased (>100%) liver TAG content without a change in circulating NEFA (Bradford et al., 2009). Our recent data demonstrates increased inflammatory markers in cows diagnosed with ketosis only and no other health disorders (i.e. the inflammation was not apparently due to mastitis or metritis). In comparison with healthy controls, ketotic cows had increased circulating LPS prior to calving and post-partum acute phase proteins such as LBP, serum amyloid A, and haptoglobin were also increased (Figure 1; Abuajamieh et al., 2016a). However, even seemingly healthy cows experience some degree of inflammation postpartum (Humblet et al., 2006). The magnitude and persistency of the inflammatory response seems to be predictive of transition cow performance (Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018). Endotoxin can originate from a variety of locations, and obvious sources in transitioning dairy cows include the uterus (metritis) and mammary gland (mastitis) (Mani et al., 2012). Additionally, we believe intestinal hyperpermeability may also be responsible for periparturient inflammation in dairy cows as many of the characteristic responses (rumen acidosis, decreased feed intake, and psychological stress) occurring during this time can compromise gut barrier function.

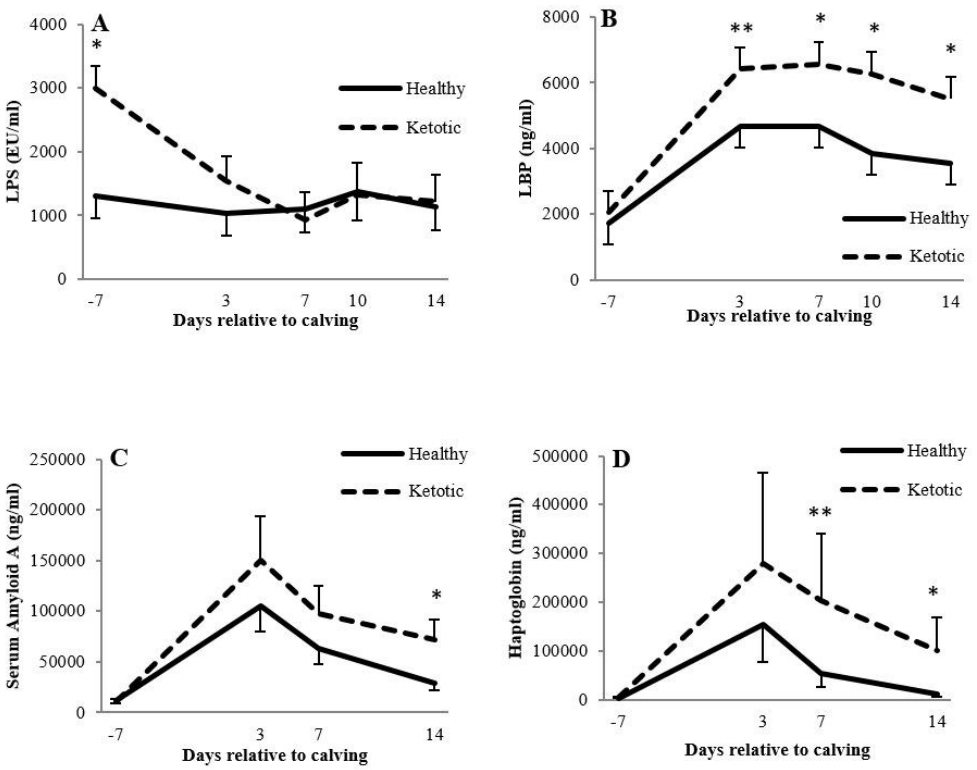

Figure 1. Markers of inflammation in healthy (solid line) and ketotic (dashed line) transition cows.

As aforementioned, mild inflammation is observed even in cows which seemingly complete the transition period successfully, suggesting that some level of inflammation plays an important role in cow health. In fact, previous reports have demonstrated that blocking endogenous inflammation (via administration of non-steroidal anti-inflammatory drugs [NSAID]) can increase the incidence of negative health outcomes (i.e., fever, stillbirth, retained placenta, metritis) and reduce productivity (Schwartz et al., 2009; Newby et al., 2013, 2017). Beneficial effects of NSAIDs have been observed on production performance (Carpenter et al., 2016), but inconsistencies exist (Priest et al., 2013; Meier et al., 2014) including how NSAIDs seemingly work better in specific parities (Farney et a., 2013) and interfere with fiber digestion (Carpenter et al., 2016) and compromise feed intake (Carpenter et al., 2017). Although NSAIDs may be an effective prophylactic strategy during the periparturient period, further research is necessary to determine the timing of administration and type and dose of NSAID that is most effective at improving health. Alternatively, administrating a chemokine (anti or even pro-inflammatory) may hold promise in improving transition cow performance.

Rumen and Hindgut Acidosis

A transitioning dairy cow undergoes a dietary shift from a high forage to a high concentrate ration post-calving. This has the potential to induce rumen acidosis (RA) as increases in fermentable carbohydrates and DMI stimulate the buildup of short chain fatty acids and lactic acid (Nocek, 1997; Enemark, 2008). Rumen acidosis has direct and

ancillary consequences accompanied by various production issues (decreased DMI, reduced milk yield, milk fat depression) and health challenges such as laminitis, liver abscesses, and potentially death (Nocek, 1997; Kleen, 2003). The mechanisms linking RA and the development of health disorders are not entirely clear, however, recent literature has indicated that inflammation associated with epithelial damage and consequential LPS translocation are at least partially responsible for production losses associated with RA (Gozho, et al., 2005; Khafipour, et al., 2009). Although many hypothesize LPS translocation occurs at the rumen epithelium directly (Guo et al., 2017; Minuti et al., 2014), others point towards LPS translocation in the hindgut to be a potential source of peripheral inflammation (Li et al., 2012). Interestingly, when RA was induced using either alfalfa pellets or high-grain diets, increased peripheral inflammation was only observed in the high-grain group, irrespective of rumen acidotic conditions being similar between the two treatments (Khafipour et al., 2009a,b). It was hypothesized that the grain supplemented group likely had increased starch flow to the hindgut, and therefore, increased fermentation that could potentially lead to hindgut acidosis and LPS translocation across the large intestine. However, we were unable to recreate production losses and systemic inflammation when we abomasally infused 500 g/d of resistant starch (Piantoni et al., 2018) or even 4 kg/d of purified corn starch (Abeyta et al., 2019). Both of our aforementioned experiments were accompanied with marked reductions in fecal pH so it is unlikely that large intestinal acidosis per se is the specific reason for systemic inflammation described in the previous reports (Li et al., 2012, Khafipour et al., 2009a,b). Regardless, we recently reported that cows with the largest decrease in fecal pH post-calving consumed less feed, produced less milk, had a larger acute phase protein response and had increased NEFA and BHB compared to cows that had a mild decrease in fecal pH following parturition (Rodriguez-Jimenez et al., 2019). Clearly, our current understanding of how hind-gut acidosis impacts the immune system and ultimately periparturient productivity is woeful.

Feed Restriction and Psychological Stress

Stress associated with feed restriction along with several other regular production practices (e.g., heat stress, weaning, transportation, overcrowding, restraint, social isolation/mixing) is frequently encountered in animal agriculture (Chen et al., 2015) and is associated with gastrointestinal hyperpermeability. In fact, we have repeatedly reported reduced intestinal barrier integrity in pigs pair-fed to their HS counterparts (Pearce et al., 2013; Sanz-Fernandez et al., 2014). Furthermore, we recently demonstrated shortened ileum villous height and crypt depth (Kvidera et al., 2017d) as well as increased appearance of the intestinal permeability marker Cr-EDTA (Horst and Baumgard, unpublished), indicating reduced intestinal health in cows fed 40% of ad libitum intake. Recent literature indicates that the corticotropin releasing factor (CRF) system may be the mechanism involved in stress-induced leaky gut (Wallon et al., 2008; Vanuytsel et al., 2014). The CRF and other members of the CRF signaling family including urocortin (1, 2, and 3) and their G-protein couple receptors CRF1 and CRF2, have been identified as the main mediators of the stress-induced intestinal changes including inflammation, altered intestinal motility and permeability, as well as shifts in ion, water, and mucus secretion and absorption (as reviewed by Rodiño-Janeiro et al., 2015). These alterations appear to

be regulated in large part by intestinal mast cells (Santos et al., 2000). Mast cells are important mediators of both innate and adaptive immunity and express receptors for the neuropeptides CRF1 and CRF2, which may in part explain the association between emotional stress and intestinal dysfunction (Smith et al., 2010; Ayyadurai et al., 2017). Furthermore, mast cells synthesize a variety of pro-inflammatory mediators (i.e., IFN-γ and TNF-α) that are released upon activation, mainly via degranulation (de Punder and Pruimboom, 2015). Excessive mast cell degranulation plays an important role in the pathogenesis of different intestinal inflammatory disorders (Santos et al., 2000; Smith et al., 2010). A better understanding of the role psychosocial stress plays on the initiation of different intestinal disorders in livestock is of obvious interest for multiple animal agriculture systems.

Metabolism of Inflammation

LPS-induced inflammation has an energetic cost which redirects nutrients away from anabolic processes that support milk and muscle synthesis (see review by Johnson 1997, 1998) and thus compromises productivity. Upon activation, most immune cells become obligate glucose utilizers via a metabolic shift from oxidative phosphorylation to aerobic glycolysis (not anaerobic glycolysis typically learned about in biochemistry classes), a process known as the Warburg effect (Figure 2).

This metabolic shift allows for rapid ATP production and synthesis of important intermediates which support proliferation and production of reactive oxygen species (Calder et al., 2007; Palsson-McDermott and O'Neill, 2013). In an effort to facilitate glucose uptake, immune cells become more insulin sensitive and increase expression of GLUT3 and GLUT4 transporters (Maratou et al., 2007; O'Boyle et al., 2012), whereas peripheral tissues become insulin resistant (Poggi et al., 2007; Liang et al., 2013). Furthermore, metabolic adjustments including hyperglycemia or hypoglycemia (depending upon the stage and severity of infection), increased circulating insulin and glucagon, skeletal muscle catabolism and subsequent nitrogen loss (Figure 3; Wannemacher et al., 1980), and hypertriglyceridemia (Filkins, 1978; Wannemacher et al., 1980; Lanza-Jacoby et al., 1998; McGuinness, 2005) occur. Interestingly, despite hypertriglyceridemia, circulating BHB often decreases following LPS administration (Waldron et al., 2003a,b; Graugnard et al., 2013; Kvidera et al., 2017a). The mechanism of LPS-induced decreases in BHB has not been fully elucidated, but may be explained by increased ketone oxidation by peripheral tissues (Zarrin et al., 2014). Collectively, these metabolic alterations are presumably employed to ensure adequate glucose delivery to activated leukocytes.

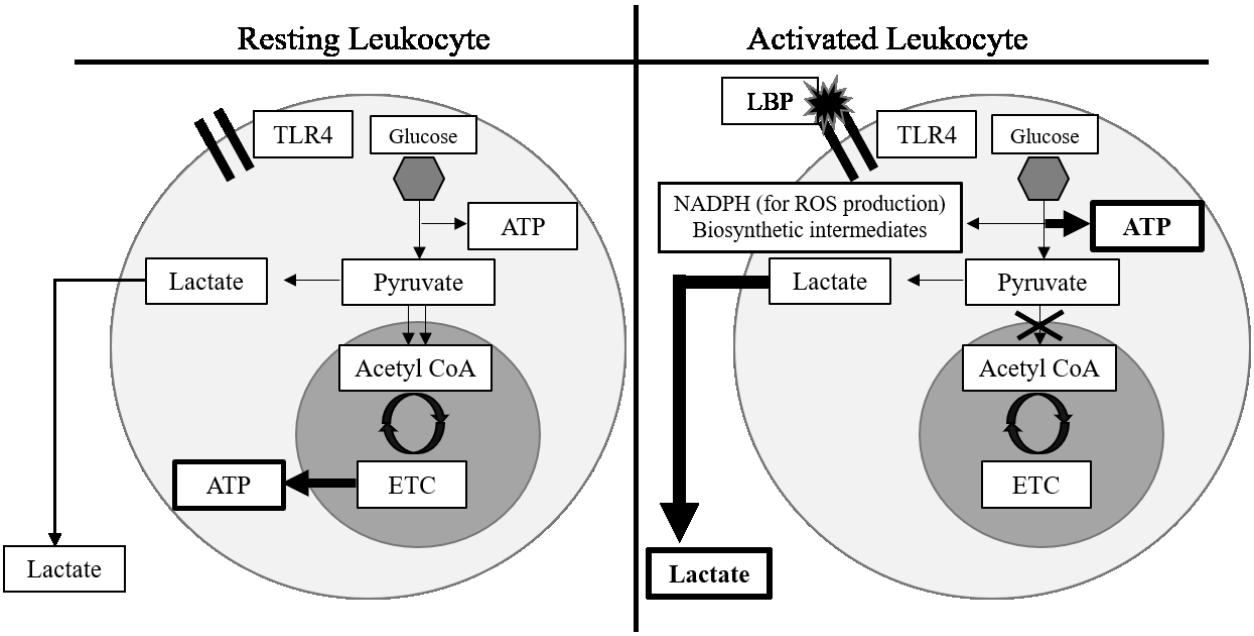

Figure 2. Metabolic pathway of a resting (A) vs. activated (B) leukocyte

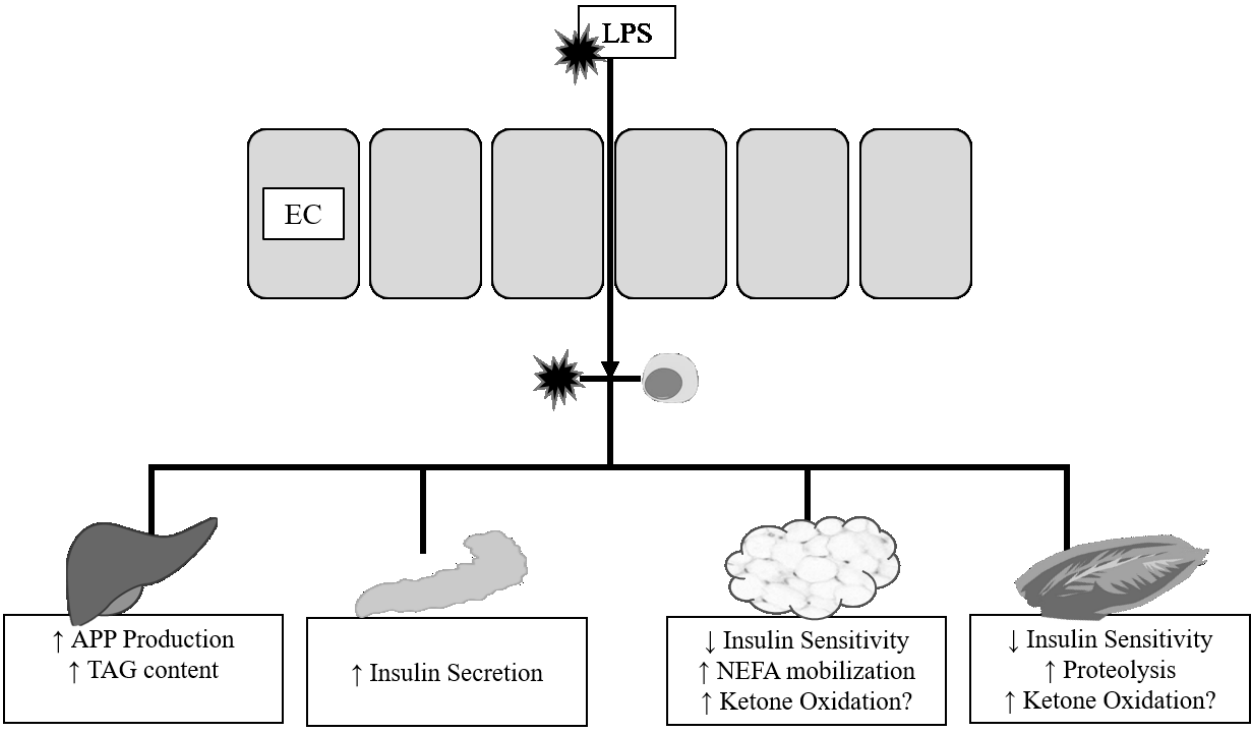

Figure 3. LPS induced alterations in peripheral metabolism.

Energetic Cost of Immune Activation

The energetic costs of immunoactivation are substantial, but the ubiquitous nature of the immune system makes quantifying the energetic demand difficult. Our group recently employed a series of LPS-euglycemic clamps to quantify the energetic cost of an activated immune system. Using this model, we estimated approximately 1 kg of

glucose is used by an intensely activated immune system during a 12 hour period in lactating dairy cows. Interestingly, on a metabolic body weight basis the amount of glucose utilized by LPS-activated immune system in mid- and late-lactation cows, growing steers and growing pigs were 0.64, 1.0, 0.94, 1.0, and 1.1 g glucose/kg $BW^{0.75}$/h, respectively; Kvidera et al., 2016, 2017b,c, Horst et al., 2018, 2019). A limitation to our model is the inability to account for liver's contribution to the circulating glucose pool (i.e., glycogenolysis and gluconeogenesis). However, both glycogenolytic and gluconeogenic rates have been shown to be increased during infection (Spitzer et a., 1985; Waldron et al., 2003b) and Waldron et al. (2006) demonstrated that ~87 g of glucose appeared in circulation from these processes. Furthermore, we have observed both increased circulating glucagon and cortisol (stimulators of hepatic glucose output) following LPS administration (Horst et al., 2019) suggesting we are underestimating the energetic cost of immunoactivation. The reprioritization of glucose trafficking during immunoactivation has particular consequences during lactation as it requires ~72 g of glucose for synthesizing 1 kg milk (Kronfeld, 1982).

Increased immune system glucose utilization occurs simultaneously with infection-induced decreased feed intake: this coupling of enhanced nutrient requirements with hypophagia obviously decrease the amount of nutrients available for the synthesis of valuable products (milk, meat, fetus, wool, etc.). We and others have now demonstrated that HS, rumen acidosis, and psychological stress increase circulating markers of endotoxin and inflammation. We believe that the circulating LPS originates from the intestine (small or large) and initiates an immune response. This activated systemic immune response reprioritizes the hierarchy of glucose utilization and milk synthesis is consequently deemphasized.

Nutritional Mitigation Strategies: The Role of Zinc (Zn) Supplementation

Potential dietary mitigation strategies aimed at improving gut health are currently of great interest, especially considering the numerous stressors (i.e., heat stress, feed restriction, acidosis) that potentially impact intestinal permeability. Zinc is an essential nutrient which is crucial for maintaining epithelial integrity (i.e., mammary, uterine, intestinal) and regulating the renewal of damaged epithelium (Alam et al., 1994). Zinc was first demonstrated to improve intestinal "health" in human leaky gut models (Alam et al., 1994; Rodriguez et al., 1996; Sturniolo et al., 2001), and we extended this to improved metrics of intestinal permeability in a variety of farm animal stress models including heat stress (Sanz-Fernandez et al., 2014; Pearce et al., 2015; Abuajamieh et al., 2016b) and feed restriction (Horst and Baumgard, unpublished using Zn hydroxychloride). Additionally, we observed altered febrile, cytokine, and acute phase protein responses during heat stress (Sanz-Fernandez et al., 2014; Pearce et al., 2015; Abuajamieh et al., 2016b; Mayorga et al., 2018) and in response to LPS administration (Horst et al., 2019) with dietary Zn supplementation. Presumably the aforementioned changes in inflammatory variables are indicative of a blunted immune response (because of improved intestinal barrier function). Therefore, Zn as a dietary supplement appears to be a promising avenue to improve gut health and to ameliorate alimentary canal associated inflammation.

Conclusion

There are various situations in an animal's life that hinder production performance (i.e., heat stress, feed restriction, rumen acidosis, etc.) and we suggest, based upon the literature and on our supporting evidence, that LPS (of intestinal origin) may be the common culprit in these circumstances. Immune activation in response to LPS markedly alters nutrient partitioning as a means of fueling the immune response. More research is still needed to understand the mechanisms and consequences of intestinal permeability and associated inflammation in order to provide foundational information for developing strategies aimed at maintaining productivity.

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Effects of Precision Essential Amino Acid Formulation on a Metabolizable Energy Basis for Lactating Dairy Cows

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Introduction

Accurate description of both nutrient supply and requirements in the dairy cow continues to be a focus of ongoing research as we work to improve the efficiency of nutrient use in high producing cattle and reduce the environmental impact of milk production. In addition, producers feel the need to optimize their cattle's performance, improving profitability through feed cost savings while complying with nutrient management. As such, areas of opportunity exist in cattle nutrition that can accomplish these objectives, particularly involving protein feeds and nitrogen (N) metabolism. Current diet formulations rely on crude protein as the metric when evaluating N supply (NRC, 2001); however, the aggregation of all N containing nutrients into one metric creates variability in predicting supply, particularly when evaluating animal performance (Ipharraguerre & Clark, 2005). Considerable progress has been made in understanding lysine and methionine requirements of lactating cattle (Rulquin et al., 1993; Schwab, 1996), and providing recommendations and demonstrating improvements in performance when animals are properly supplied with these amino acids (Armentano et al., 1997; Noftsger & St-Pierre, 2003). The same efforts that went into the Met and Lys requirements and supply should be applied to other essential amino acids (EAA), calling for the abandonment of crude protein and the move towards a more accurate representation of N supply on an amino acid basis.

In an effort to address this approach, changes have been made to the most recent research version of the CNCPS v.7 (Higgs et al., 2014), disaggregating crude protein into its constituents and accounting for these on a N basis. The implications of the changes allow for more accurate predictions of rumen N and amino acid supplies to the cow, particularly when coupled with the estimation of endogenous protein flows (Ouellet et al., 2007; Marini et al., 2008) and updated estimations of amino acid requirements and efficiency of use (Lapierre et al., 2007; Lapierre et al., 2012). Work has been conducted to evaluate CNCPS v.7 performance when balancing diets for both rumen N requirements and EAA supply (Higgs et al., 2014). Findings from that study indicated that notwithstanding lower levels of crude protein (< 14% DM) in the diet, cattle maintained a high level of performance when supplied with adequate rumen N and balanced for EAA. Further investigation eluded to a potential relationship between the supply of digestible EAA and the supply of metabolizable energy (ME) in the diets fed. This loglogistic relationship (Figure 1) was demonstrated when the ratio of AA required (AAR) to AA supplied (AAS) was regressed against digestible AA supply relative to Mcals of ME. To further expand on these relationships, optimum points of digestible AAS relative to ME can be estimated by regressing predicted AAR on the digested AAS (figure not shown).

Solving for the upper critical level of the second order derivative of that regression (Doepel et al., 2004), determined the efficiency of use of each EAA , and interpolating that efficiency provides a solution for the supply of EAA per unit of ME at the optimum efficiency of use for productivity (Figure 1). This technique can be applied to calculate the requirements for all EAA and the supply of each AA relative to ME, on a gram basis, can be calculated.

The recognition of protein and energy's interrelationship is not a novel idea, particularly when discussing mammalian metabolism. Metabolic flexibility, particularly in the mammary gland, allows dairy cattle to meet their energetic needs through either the use of high yielding energy substrates or N containing compounds (i.e. amino acids) when other substrates are lacking (Lobley, 2007). Studies have demonstrated that the supplementation of both propionate and casein have a greater, additive effect on milk yield in both cattle (Raggio et al., 2006) and lactating sows (Dunshea et al., 2005) than if either one was solely supplemented. In spite of the collinearity of these two types of nutrients, their relationship seems more prevalent when exploring the relationship between digestible EAA and metabolizable energy (Higgs et al., 2014). Further, nearly all of AA supply can be related energy when swine diets are formulated (NRC, 2012). Depending upon the stage of life, the weight of animal, and it's production (meat or milk), the Swine NRC provides specific tables containing ideal amino acid profiles for a given animal which can then be related to a recommended energy content of the diet.

With this in mind, the objective of this study was to evaluate the approach in lactating cattle using CNCPS v.7 to formulate diets adequate in rumen N and balanced for EAA relative to the ME supply. Our hypothesis was that the efficiencies of use for each EAA determined by Higgs (2014) and Higgs and Van Amburgh (2016) are the optimum efficiencies and we when related to energy, the requirements can be calculated on a gram basis and that pending upon the results of this study, will either be modified or reinforced. The hypothesis involved testing the ranges in the grams of digestible AA required per unit of ME (Figure 1). Those ranges represent the upper and lower limits of each EAA observed in the data sets and the hypothesis involved evaluating the limits as a sensitivity test of the concept.

Methodology

Accurate description of feed chemistry for all ingredients included in this experiment was of the utmost importance when considering this study's validity. Protein feed samples obtained from a commercial feed mill (Purina Animal Nutrition, Caledonia, NY) and forage samples from the Cornell University Ruminant Center were screened for chemical composition. Of particular interest was the quantification of total N, N digestibility, and amino acid profiles for all feeds. Quantification of total N was obtained via the Leco total combustion method (Leco, St. Joseph, MI). Amino acid profiles from parallel laboratory experiments (Van Amburgh et al., 2017) were adapted and matched to fit the analyzed feeds. Upon completion, both feed chemistry results and animal inputs were implemented within CNCPS v.7 for diet formulation.

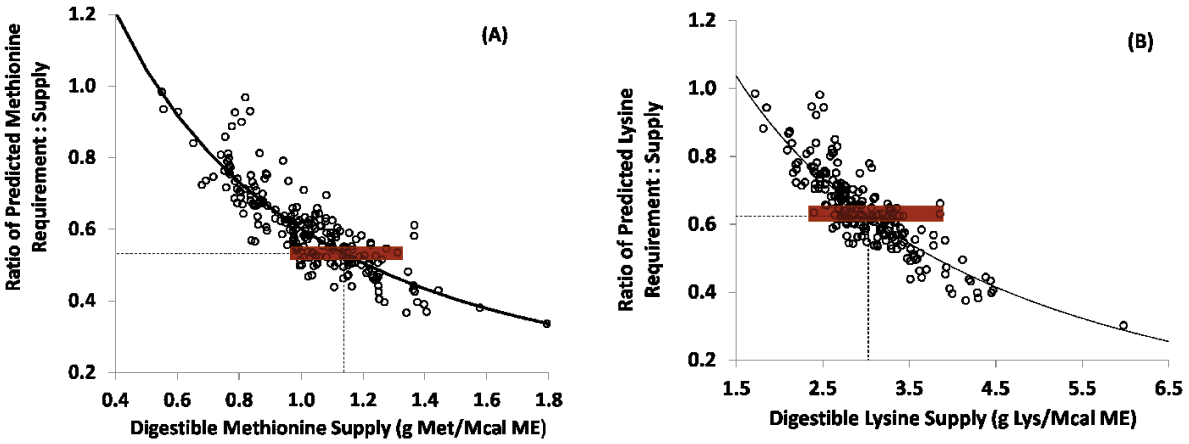

Figure 1. Relationship between model predicted EAA requirement: supply and EAA supply relative to ME for Met (A) and Lys (B). The dashed line in (A) & (B) represents the Met or Lys supply at the optimum ratio of model predicted Met or Lys requirement and supply. The red bar represents ± 1 standard deviation of AA supply relative to ME supply.

Dietary treatments within this experiment were based on previous results exploring amino acid balancing in lactating dairy cattle (Higgs, 2014; Higgs and Van Amburgh, 2016). Findings from this study suggest an optimal requirement of each EAA at a given level of metabolizable energy (Figure 1; shaded rectangles); however, variation exists around data, creating ambiguity about their accuracy. In an effort to confirm the values, three diets were formulated to be isocaloric and excess in energy as a means to prevent a first-limiting effect on animal performance. The only differences in these diets were in the level of EAA fed, creating differences in the ratios of EAA to metabolizable energy. The Neutral diet (NEU) was formulated to match the optimal ratios determined by Higgs (2014) and Higgs and Van Amburgh (2016), whereas the Positive (POS) and Negative (NEG) control diets were formulated to be one standard deviation above and below the optimal ratio for each EAA (Table 1).

Thus, the three diets were isocaloric, varied only in the level of EAA fed and the rumen N balance was positive for all diets through the use of additional urea when need to ensure the rumen N balance was always positive. Protein feeds were evaluated for intestinal digestibility using the assay of Ross et al. (2013) to ensure EAA availability in formulation and this information was considered to ensure the grams of each EAA met the formulation criteria. Cattle on the Neutral diet (14.5% crude protein [CP]) were fed according to previously calculated optimal grams of AAS to ME (Higgs, 2014), whereas the Positive (16% CP) and Negative diets (13.5% CP) were formulated for ± 1 standard deviation relative to the Neutral diet, respectively for all EAA (Figure 1; Table 1).

The experiment was conducted at the Cornell University Ruminant Center (Harford, NY) from July - December 2018. All procedures involving animals were approved by the Cornell University Institutional Animal Care and Use Committee. One hundred and forty-four (n=144) Holstein cows [26 primiparous and 118 multiparous; 2.9 ± 1.4 lactations; 92 ± 24 DIM at enrollment] were enrolled in a 114 day longitudinal study. Two enrollments (96 cattle in enrollment 1 [July – November 2018] and 48 cattle in

enrollment 2 [August – December 2018) periods were necessary to maintain the relevant period of lactation for observation. Cattle were blocked into 16 cow pens and balanced for parity, DIM, previous lactation performance, and current body weight. Cattle were housed in a freestall setting at stocking density of 100%. Each pen was fed TMR once daily at approximately 0600 h and pens were targeted for 5% refusal rate. All nine pens were fed the POS diet during a 14 day covariate period and randomly assigned to one of three diets described above for the remaining 100 d. Covariate samples were taken in the second week of the period to allow animals to acclimate to their new environment and diet.

Table 1. The predicted AA supply for each diet compared with the calculated optimum supply (g digested AA/Mcal ME)

	Negative[1]		Neutral		Positive	
g AA/Mcal ME	Formulated	Target	Formulated	Target	Formulated	Target
Arg	2.01	2.03	2.25	2.04	2.30	2.32
His	0.88	0.79	0.98	0.91	1.17	1.03
Ile	2.08	1.86	2.27	2.16	2.24	2.45
Leu	3.24	2.95	3.54	3.42	4.00	3.89
Lys	2.84	2.62	3.00	3.03	3.49	3.44
Met	1.01	0.98	1.09	1.14	1.29	1.30
Phe	2.15	1.86	2.30	2.15	2.54	2.44
Thr	2.03	1.85	2.26	2.14	2.40	2.43
Trp	0.65	0.51	0.68	0.59	0.62	0.67
Val	2.27	2.14	2.51	2.48	2.76	2.82
Lys:Met	2.65	2.67	2.75	2.66	2.70	2.65

[1] Negative = All EAA scaled one standard deviation below ideal EAA ratio according to Higgs (2014); Neutral = All EAA scaled to ideal EAA ratio according to Higgs (2014); Positive = All EAA scaled one standard deviation above EAA ratio according to Higgs (2014). All diets balanced and in excess of ME.

Body weight and body condition score (1-5 scale) were measured and recorded weekly for all cattle. Cattle were milked three times daily (0600, 1400, and 2200 h) with milk weights recorded at every session (Del Pro Farm Manager; De Laval). Milk samples were collected weekly during three consecutive milkings and analyzed for fat, true protein, lactose, total solids, and MUN (Dairy One, Ithaca, NY). Milk component yield was calculated as the sum-product of daily milk yields at each session throughout a given week and the analyzed component values of the same week. Energy corrected milk was calculated according to Tyrrell and Reid (1965). Dry matter intake was determined daily for each pen as the difference between feed offered and refused (FeedWatch; Valley Ag Software). Samples of TMR and refusals were sampled twice each week, composited, and analyzed for nutrient composition using near infrared reflectance spectroscopy. Forage samples were collected weekly and sent for wet chemistry analysis of chemical components. Additionally, mix ingredients included in the grain mix were collected whenever new batches of grain were delivered to the farm and analyzed by wet chemistry for chemical composition.

Blood samples were taken from cattle every other week throughout the experiment. Cattle were bled at least 4 hours following feeding from the coccygeal vein into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). Plasma samples were subjected to plasma urea N (PUN) analysis via enzymatic colorimetric assay.

A sub-sample of six 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 six 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 chemistry results were produced via PROC MEANS to provide mean, standard deviation, and standard error of all feed components analyzed. Continuous measurements which were not repeated over time were subjected to ANOVA (PROC MIXED) with fixed effects including enrollment and dietary treatment. Measurements taken over time were subjected to repeated measures ANOVA (PROC MIXED) and included fixed effects of enrollment, dietary treatments, time, and the interaction of dietary treatment and 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

Dietary ingredients and chemical composition of the three diets fed throughout the experiment are presented in Table 2. Small differences between the formulated and observed levels of CP were observed, specifically for cattle fed the NEG diet (13.5% formulated; 14.0% observed); however, the relative differences in N and EAA supply among treatments was still maintained throughout the experiment. Diets did maintain their isocaloric formulation among treatments and throughout the experiment. All other chemical components in the diet remained relatively similar among treatments and throughout the experiment. As indicated earlier, urea was formulated into all three diets to maintain adequate rumen N levels. One of the structural differences in CNCPS v7 is the ability to separate the rumen N requirements from the post-ruminal EAA requirements and in doing so, makes the process of formulating for EAA more accurate as the N can be partitioned more effectively.

Daily supply of EAA as predicted by CNCPS v.7 are shown in Table 4. Since the objective of the study involved creating isocaloric diets while varying the ratio of EAA to ME, the supply of EAA delivered to cattle had to move in a stepwise fashion. As shown in the table, most EAA (Arg, His, Leu, Lys, Met, Phe, Thr, and Val) increased in supply as the N supply increased in the three diets; however, two EAA (Ile and Trp) did not show this same trend. Ideally, the supply of all EAA would increase in such a way to match the objective of this study. Realistically, both the availability of feed ingredients with a given

profile of AA and the variability of feed chemistry for the availale feeds make it a difficult process to have the supply increase in all EAA when moving from the negative to the positive treatment. At this point, we do not believe the lack of Ile and Trp supply had a major effect on the results of the study. Isoleucine is classified as a Group 2 AA and the mammary takes up this AA in excess of output, particularly for the creation of non-essential AA (NEAA) or substrates used in the creation of lactose (Lobley, 2007). Little is known about the supply of tryptophan and milk performance of dairy cattle. Within this study, the average supply of His in all three diets is 90% of the supplied level of Met. Previous literature has suggested that the supply of His should match, or exceed (up to 110% of Met supply) to allow for an optimal response in animal performance (Lee et al., 2012). Further work should be done to evaluate the response of His supplementation in the context of a study similar to the one presented here.

Dry matter intake was not different among the treatments and remained relatively stable throughout the experiment (Table 4; Effect of time not shown). Differences were observed in both milk yield and energy correct milk values, where NEG cattle yielded less milk than both the NEU and POS cattle. Whereas cattle fed, the NEU and POS diets were not significantly different between each other. Similar trends were observed for component yields. This suggests that the increased supply of EAA from the NEG to the NEU had a greater impact on milk yield and component output than the difference in supply from NEU and POS, indicating marginal effectiveness. This apparent limit is further demonstrated, as MUN output is significantly different in each diet, increasing from NEG to POS, suggesting that cattle in the POS did not need the additional EAA supplied in that diet. Overall, this demonstrates a plateau effect of EAA supply indicating the profile and amount of EAA supplied to the cattle fed the NEU diet were adequate for the expected milk yield.

Initial and final body weights were not different among treatments and all treatments were able to provide nutrients for weight gain throughout the experiment. Initial body condition score was not different among treatments and even through final condition scores were greater for NEU and POS compared to NEG, the numerical differences are negligible. Evaluation of feed efficiency indicated that NEU and POS animals were more efficient than NEG. These observations are extended to the N use efficiency where the NEG treatment had the lowest efficiency whereas there was no difference between NEU and POS treatments. Not surprisingly, daily metabolizable protein intake, as predicted by the CNCPS, was different for all treatments and increased in a stepwise fashion. What is interesting is that NEU and POS performed in a similar capacity, despite a 200-gram difference in metabolizable protein supply. This strongly suggests that the MP supply does not fully describe the N requirements of lactating cattle and that refining the diets on a digestible EAA basis, provides more accuracy. A figure summarizing these observations is presented to demonstrate animal performance when given varying ratios of digestible EAA/ME and further highlights the waning effect of increasing EAA supply relative to a fixed level of ME, particularly between NEU and POS diets (Figure 2).

Table 2. Ingredients and chemical composition of experimental diets

Ingredient, % DM	Negative[1]	Neutral	Positive
Corn silage	51.49	51.49	50.40
High moisture ear corn	9.43	9.46	9.93
Triticale	7.25	7.25	7.98
Corn grain	6.38	6.42	5.95
Soybean meal	8.16	5.55	2.72
Soybean hulls	9.25	3.84	2.83
SoyPLUS[2]	--	0.91	3.59
Canola	1.81	9.17	6.31
Urea	0.62	0.51	0.51
Smartamine M[3]	--	0.04	0.05
Smartamine ML[4]	--	--	0.07
Blood meal	--	--	3.08
Energy Booster	0.73	0.73	0.91
Dextrose	1.63	1.63	2.18
Minerals and Vitamins	3.26	2.90	3.15
Chemical components[6], % DM			
CP	14.04	14.75	15.95
SP, % CP	42.93	40.29	37.33
Ammonia, % SP	13.53	14.57	12.67
ADICP, % CP	5.68	5.86	5.46
NDICP, % CP	15.01	15.47	18.66
Acetic acid	0.45	0.45	0.46
Propionic acid	0.02	0.02	0.02
Lactic acid	2.57	2.58	2.61
Sugar	3.95	4.06	3.90
Starch	29.82	29.31	29.30
Soluble fiber	6.01	5.55	5.05
ADF	20.79	19.96	19.77
NDF	32.39	31.03	31.36
Lignin, % NDF	8.06	9.65	8.73
$uNDF_{240}$, % NDF	25.50	29.09	28.73
Ash	6.60	6.92	6.57
EE	3.49	3.61	3.78
Metabolizable Energy, Mca/kg	2.58	2.60	2.61

[1] Negative = balanced for ME (assuming 45 kg ECM), all EAA scaled one standard deviation below ideal EAA ratio according to Higgs (2015); Neutral = balanced for, all EAA scaled to ideal EAA ratio according to Higgs (2015) ; Positive = balanced for ME, all EAA scaled one standard deviation above EAA ratio according to Higgs (2015)

[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. SP = soluble protein; ADICP = CP insoluble in acid detergent; NDICP = CP insoluble in neutral detergent; WSC = water soluble carbohydrates; uNDF240 = undigested NDF after 240 hours of in vitro fermentation; EE = ether extract.

Table 3. Daily supply of essential amino acids for each treatment diet.

AA, grams	Negative[1]	Neutral	Positive
Arg	143.14	161.04	164.43
His	62.78	70.42	83.81
Ile	147.85	162.37	160.56
Leu	229.92	253.31	286.27
Lys	201.70	222.12	250.07
Met	71.44	78.30	92.67
Phe	153.00	164.71	181.63
Thr	144.43	161.78	171.85
Trp	45.92	48.93	44.66
Val	161.01	179.55	197.46

[1] Negative = All EAA scaled one standard deviation below ideal EAA ratio according to Higgs (2014); Neutral = All EAA scaled to ideal EAA ratio according to Higgs (2014); Positive = All EAA scaled one standard deviation above EAA ratio according to Higgs (2014). All diets balanced and in excess of ME.

Table 4. Effects of treatment diets on milk production, intake, body weight and body condition scores.

	Negative[1]	Neutral	Positive	SEM	Treatment
Intake and milk production, kg/d					
Dry matter intake	27.9	28.2	28.5	0.27	0.98
Energy correct milk yield[2]	40.5^{a}	43.7^{b}	44.8^{b}	0.57	<0.01
Milk yield	36.8^{a}	39.8^{b}	40.8^{b}	0.47	<0.01
True protein yield	1.13^{a}	1.26^{b}	1.28^{b}	0.01	<0.01
Fat yield	1.53^{a}	1.62^{ab}	1.67^{b}	0.03	<0.01
Lactose yield	1.77^{a}	1.91^{b}	1.97^{b}	0.03	<0.01
Milk composition, %					
True protein	3.09^{a}	3.17^{b}	3.14^{b}	0.02	<0.01
Fat	4.20	4.12	4.14	0.06	0.64
Lactose	4.78	4.82	4.81	0.02	0.31
MUN	10.5^{a}	11.4^{b}	13.8^{c}	0.14	<0.01
Body weight and condition					
Initial Body Weight, kg	691.5	692.7	697.5	4.27	0.83
Final Body weight, kg	721.2	718.2	723.3	3.26	0.09
Body weight change, kg/wk	2.26	2.03	2.53	0.33	0.58
Initial BCS, 1-5 Scale	2.90	2.86	2.84	0.02	0.75
BCS, 1-5 scale	2.88^{a}	2.92^{b}	2.93^{b}	0.01	0.01
CNCPS v.7 Parameters					
Feed Efficiency	1.48^{a}	1.55^{b}	1.59^{b}	0.02	<0.01
Metabolizable Protein Intake, g/day	2656.6^{a}	2974.4^{b}	3207.5^{c}	162.4	0.02
Nitrogen Use Efficiency	0.282^{a}	0.300^{b}	0.299^{b}	0.003	<0.01

[1] Negative = All EAA scaled one standard deviation below ideal EAA ratio according to Higgs (2014); Neutral = All EAA scaled to ideal EAA ratio according to Higgs (2014); Positive = All EAA scaled one standard deviation above EAA ratio according to Higgs (2014). All diets balanced and in excess of ME.

[2] Estimated according to Tyrrell and Reid (1965)

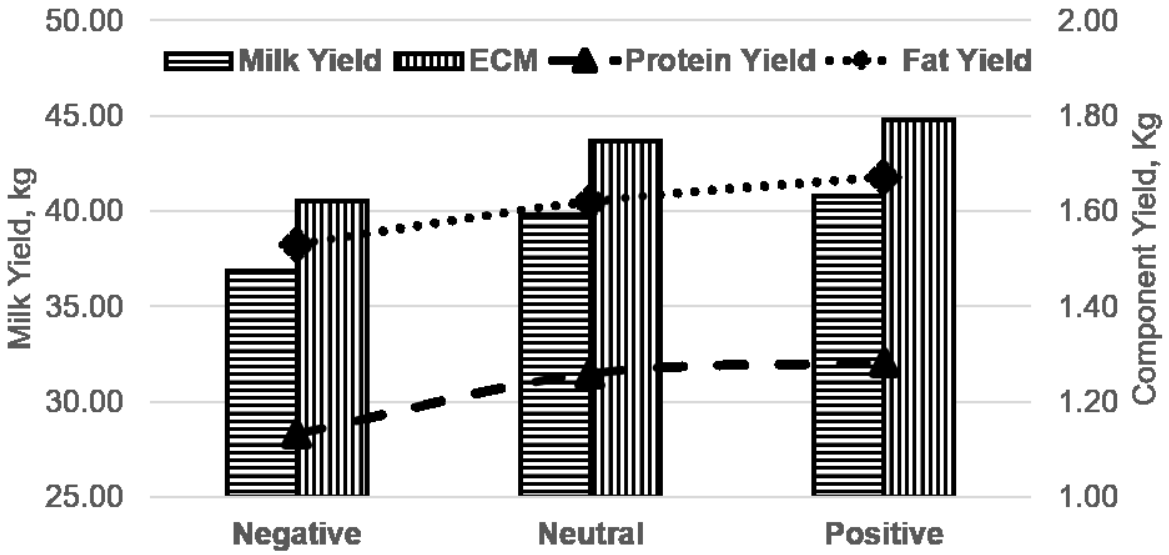

Figure 2. Effect of dietary treatment on milk, energy corrected milk, and component yield for animals fed.

In summary, cattle fed the NEU dietary treatment produced similar levels of energy corrected milk and yield similar production of fat components when compared to cattle fed the POS treatment (Table 4; Figure 2). The productivity of the cattle was similar even though the difference in crude protein of the two diets was over 1 units, suggesting that cattle fed the NEU diet were at least as productive with their N supply as cattle fed the POS diet. Evaluation of MUNs indicate that the excretion of urea nitrogen was higher in the POS diet over the NEU diet, suggesting either that NEU cattle may have had a more balanced profile of EAA or that they were less wasteful with the N given to them. Cattle fed the NEG likely had a deficient supply of EAA as their production and feed efficiency was lower than either the NEU or POS cattle. Further analysis of the data collected from this experiment, coupled with model evaluation through CNCPS v.7, will help to reinforce our hypothesis that the optimum digestible EAA supply relative to ME generated by Higgs (2014) were within the range of true requirements for lactating cattle. The results from this study will be used to formulate diets for a similar study in which cattle performance will be evaluated using a fixed N and AA supply while varying levels of rumen fermentable carbohydrates to stimulate propionate and thus, lactose production.

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Short chain fatty acid absorption and regulation of ruminal pH[1]

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Introduction

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

Mechanisms of SCFA Absorption and the Linkage to Ruminal pH

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

SCFA Absorption

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

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arrangement of the ruminal epithelium (Steven and Marshall, 1980). The outer-most layer of the ruminal epithelium is the stratum corneum. The cells within the stratum corneum are highly keratinized and are not metabolically active. These protect the underlying strata from physical abrasion, but likely contribute very little as a barrier or promoting factor for SCFA absorption. The next layer is the stratum granulosum. This stratum is characterized by cells that are increasingly keratinized and have few intracellular organelles. However, the stratum granulosum is the primary site for tight-cell junctions and thus acts as a physical barrier to ensure absorption of SCFA occurs while preventing passage non-desired molecules. The next layer is the stratum spinosum. The cells within the stratum spinosum are metabolically active and have gap junctions that serve to facilitate cell-to-cell communication and exchange of ions. Finally, the most inner layer is the stratum basale. The stratum basale are highly active cells and are the layer where cell division occurs. The cell division is necessary to provide new cells that mature and differentiate as cells migrate toward the stratum corneum.

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

While mechanisms of SCFA have been investigated since the 1940's (Danielli et al., 1945; Masson and Phillipson, 1951; Ash and Dobson, 1963), it was largely argued that SCFA absorption occurred via passive diffusion. The suggestion for passive diffusion was based on the inability to achieve saturation of SCFA absorption with increasing SCFA concentration and that reducing pH increased SCFA absorption (Dijkstra et al., 1993; López et al., 2003; Graham et al., 2007). It is important to recognize that as pH declines, the proportion of SCFA in the undissociated state increases and that only undissociated SCFA are permeable to cross the lipid bilayer of cells (Walter and Gutknecht, 1986; Gäbel et al., 2002). Thus, a reduction in pH would increase the proportion of undissociated SCFA that could then freely diffuse across the rumen epithelium.

There are numerous theoretical constraints for a model solely relying on passive diffusion. Firstly, the proportion of SCFA in the undissociated state (pKa = 4.8) is low under normal pH conditions in the rumen. Even with pH values of 5.8, more than 90% of the SCFA would be in the dissociated state. Previous researchers had suggested that there was an acidic pH microclimate on the luminal side of the ruminal epithelia (Graham and Simmons, 2005) allowing for a greater proportion of SCFA in the undissociated state immediately adjacent to the epithelium. However, basic apical pH values have been reported (7.47 to 7.68) depending on the incubation conditions (Leonhard-Marek et al., 2006). Lipophilicity constants also suggest that butyric acid should be absorbed about 14 times more rapidly than acetic acid (Walter and Gutknecht, 1986). However, similar fractional absorption rates have been reported among SCFA in vitro (Aschenbach et al., 2009) and when differences are found (Dijkstra et al., 1993; López et al., 2003), they are

not consistent with the increase predicted based on lipophilicity. Moreover, a recent study showed that although the concentration of SCFA increased from 10 to 50 m*M*, the rates of acetate and butyrate absorption only increased by 2.1 and 2.4 times for acetate and butyrate, respectively (Schurmann et al., 2014). The model of passive diffusion also does not consider how SCFA are transported across the basolateral (blood facing) side of the epithelium.

A simplified model showing the current understanding of the mechanisms involved in SCFA absorption and how the absorption of SCFA contributes to the stabilization of ruminal pH is depicted in Figure 1 (Aschenbach et al., 2011). The predominant mechanisms include: 1) $SCFA^-/HCO_3^-$ anion exchange; 2) passive diffusion; 3) nitrate-sensitive SCFA absorption; 4) proton-coupled $SCFA^-$ transport; and 5) electrogenic SCFA transport. While these are the major absorption mechanisms, other processes such as Na^+/H^+ exchange, and bicarbonate import into the cells are required to enable the maintenance of intracellular pH and to promote SCFA absorption. These pathways have been reviewed extensively in Aschenbach et al. (2011).

Much of the SCFA absorption occurs through anion exchange where $SCFA^-$ are absorbed in exchange for release of HCO_3^- into the rumen and further the $SCFA^-$ can cross the basolateral membrane in exchange for HCO_3^- import into the cell (Bilk et al., 2005; Aschenbach et al., 2009; Penner et al., 2009b). Based on the available data, approximately 42 to 57%, 0 to 14%, and 29 to 59% of the acetate transport relies on bicarbonate-dependent, nitrate-sensitive, and passive diffusion, respectively (Penner et al., 2009a; Schurmann, 2013). For butyrate, the proportion accounted for by bicarbonate-dependent transport, nitrate-sensitive transport, and passive diffusion are 24 to 46, 0 to 4, and 25 to 76%, respectively (Penner et al., 2009a; Schurmann, 2013).

In the rumen, the vast majority of the SCFA will be in the dissociated state (**$SCFA^-$**). Absorption of $SCFA^-$ occurs in exchange for HCO_3^- in an electro-neutral process that is mediated by a number of anion exchangers (Bilk et al., 2005; Aschenbach et al., 2009; Penner et al., 2009b). This mechanism provides a source of bicarbonate to the ruminal environment where it can neutralize a proton via the carbonic anhydrase reaction producing carbon dioxide and water. Driving forces for bicarbonate-dependent transport include the concentration of ruminal SCFA and ruminal pH. In fact, the bicarbonate-dependent SCFA absorption increases with increasing luminal SCFA concentration and with decreasing ruminal pH (Aschenbach et al., 2009). The bicarbonate facilitating this transport does not seem to occur in the cytosol, but rather is transported from arterial circulation into the cell (Sehested et al., 1999; Aschenbach et al., 2009). There are several bicarbonate transporters including anion exchangers on the basolateral (blood-facing) side that may also help to export $SCFA^-$ out of the cell and into arterial blood. Thus, it appears that this transport process is crucial in terms of helping to regulate ruminal pH (Penner et al., 2009a) and exporting SCFA to be metabolized by other tissues.

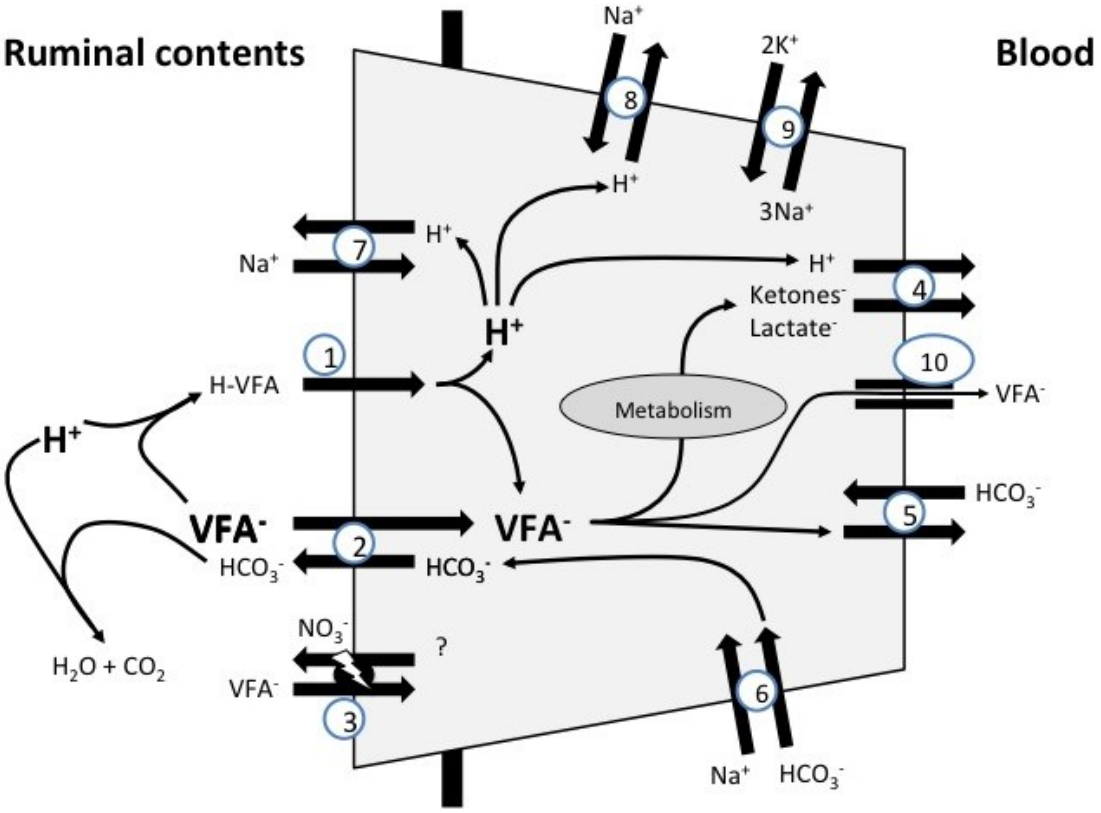

Figure 1. Partial model depicting the current understanding for SCFA absorption in relation to the stabilization of ruminal pH. 1) Diffusional absorption of SCFA facilitates the removal of a proton associated with the SCFA. This proton will rapidly dissociate in the cytosol where it can be exported by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

When H-SCFA are absorbed via passive diffusion, 1 proton is removed from the ruminal digesta; however, H-SCFA will dissociate in the cytosol releasing $SCFA^-$ and H^+. The proton (H^+) released must be removed from the cell or neutralized in order to maintain intracellular pH and tissue integrity. Transporters involved in the regulation of intracellular pH include the sodium/hydrogen exchangers **(NHE)** that export protons back to the lumen

or into extra-cellular spaces. In addition to NHE, the monocarboxylate transporter **(MCT)** has been shown to be localized on the basolateral membrane (blood facing; Graham and Simmons, 2007) and can facilitate the removal of a proton along with metabolic end-products of SCFA metabolism such as ketone bodies and lactate (Müller et al., 2002; Kirat et al., 2006). Thus, the direction of proton export has major implications for whether passive diffusion contributes to the stabilization of ruminal pH. For example, if the proton is exported back into the rumen contents as a strategy to maintain intracellular pH, there would be no net proton removal from the rumen and therefore ruminal pH would not be affected. Interestingly, the expression and activity of NHE in ruminal epithelia increase when highly fermentable diets are fed (Etschmann et al., 2009; Yang et al., 2009; Schurmann, 2013). However, due to the complexity of the transport mechanisms involved and the regulation of their activity, it is very difficult to quantify or predict the proportion of protons recycled back to the lumen relative to those that account for permanent removal from the ruminal contents. That said, it is clear that under some circumstances passive diffusion does contribute to the removal of protons from the rumen (Penner et al., 2009a).

In addition, it is now known that there is a nitrate-sensitive transport pathway for SCFA. This process occurs both in the presence and absence of bicarbonate (Aschenbach et al., 2009), but currently the transporters involved are not known. Recent unpublished work (K. Wood, J.R. Aschenbach, F. Stumpff, and G.B. Penner) has shown a clear inhibitory effect with increasing concentrations of nitrate for acetate but no effect for butyrate. Future studies are required to improve our understanding of this transport mechanism and its regulation. Finally, electrogenic $SCFA^-$ transport has been documented (Stumpff et al., 2009; Georgi et al., 2013). This transport process is thought to be mediated by maxi-anion channels but the total contribution to SCFA transport is not currently known.

Evidence Linking SCFA Absorption to the Stabilization of Ruminal pH

Early studies (Masson and Phillipson, 1951; Dobson and Ash, 1963; Gäbel et al., 1991) had suggested that SCFA absorption could be one mechanism for the stabilization of rumen pH. However, the first evidence supporting the pH stabilizing effect of SCFA absorption was provided by Resende Júnior et al. (2006). In that study moderate (r^2 = 0.43) positive correlations between the fractional rate of SCFA clearance and ruminal pH were observed suggesting that greater rates of SCFA clearance corresponded to improved ruminal pH. Resende Júnior et al. (2006) further evaluated whether the effect on pH was due to absorption of SCFA across the rumen wall or the passage of SCFA out of the rumen finding that both mechanisms were positively related to ruminal pH. In another study Penner et al. (2009b), reported negative associations between the expression of a number of genes involved in SCFA metabolism and the severity of ruminal acidosis for dairy cows fed a diet containing 64% concentrate. While these studies (Resende Júnior et al., 2006; Penner et al., 2009b; Schlau et al., 2012) showed relationships between ruminal pH or the severity of ruminal acidosis and the absorption of SCFA or indicators for intra-epithelial metabolism of SCFA, they cannot prove that SCFA absorption improves ruminal pH nor can they elucidate how the pathway of SCFA and type of SCFA affect ruminal pH.

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

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

Nutritional Modulation of SCFA Transport

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

Low Feed Intake and Feed Deprivation Decrease SCFA Absorption

The vast majority of current research has focused on rumen epithelial adaptation from an anabolic perspective, however, in times of scarcity or in response to a nutritional insult, the adaptive response certainly includes regression. In fact, the long-term changes induced by a low plane of nutrition have been shown to decrease gut mass and reduce O_2 consumption by visceral tissue, and reduce SCFA absorption (Doreau et al., 1997). Understanding how the ruminal epithelium responds to reductions in SCFA exposure due to a transient low feed intake and, more importantly, the timeline required for the epithelium to return to the pre-restriction function is needed to develop feeding strategies and mitigate disorders associated with digestive upset.

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

A rapid reduction in ruminal epithelial function may be a compensatory mechanism to reduce energy expenditure by ruminal tissue (Zhang et al., 2013a) during periods of low energy intake. However, given the transient nature of low feed intake under

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

Ruminal Acidosis Compromises SCFA Absorption

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

While the total SCFA absorption rate was not different between the short- and long-adapted cattle, it was very clear that induction of ruminal acidosis decreased SCFA absorption (Schwaiger et al., 2013a,b) when measured 2 d following induction of ruminal acidosis but not when measured 9 d after the induction of ruminal acidosis. Moreover, there appears to be a compensatory shift in ruminal buffering strategies such that absorption is reduced following a bout of ruminal acidosis while at the same time, saliva production increases. Thus, it appears that ruminal acidosis impairs SCFA absorption but the recovery following a bout of ruminal acidosis may be rapid and that cattle may increase salivary buffer supply to compensate for the reduction in SCFA absorption. The

negative effect of severely low ruminal pH on SCFA absorption is supported by previous work in vivo (Krehbiel et al., 1995) and in vitro (Wilson et al., 2012).

Promoting SCFA Absorption

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

Dietary Fatty Acid Supply and Composition Affect SCFA Absorption

Dietary fatty acids are often used to increase energy density of the diet and can modulate composition of tissues (Owens and Gardner, 2000). However, we are not aware of any studies that have evaluate whether ruminal epithelial composition can be manipulated and whether changes in composition affect SCFA absorption. Twenty-one Holstein steers (194 ± 10.7 kg) were randomly assigned to the control (**CON**; contained

2.2% ether extract) or one of two lipid treatments (contained 5% ether extract) utilizing saturated (**SAT**) or unsaturated sources (**UNSAT**) of lipid (Verdugo and Penner, unpublished). The SAT lipid sources were primarily from tallow and palmitic acid while the UNSAT was provided from flax and megalac. After 30 d, calves were killed and samples of ruminal digesta, blood, and ruminal tissue were collected for fatty acid analysis and ruminal tissues were also used for ex vivo measurement of acetate, propionate, and butyrate uptake and flux. WE observed that inclusion of lipid increased ($P < 0.01$) the concentration of fatty acids in ruminal fluid, but SAT and UNSAT did not differ. Feeding UNSAT decreased the proportion of saturated FA and increased the proportion of mono and polyunsaturated fatty acids in ruminal fluid. The changes in ruminal fluid were also reflected in plasma and ruminal tissue. The ruminal epithelial concentration of fatty acids tended ($P = 0.10$) to be greater for calves fed lipid and for calves fed UNSAT vs. SAT ($P = 0.06$). Interestingly, calves fed supplemental lipid had greater ($P = 0.03$) butyrate uptake than CON, and butyrate uptake was 44% greater for SAT than UNSAT ($P < 0.01$) suggesting that fatty acid supply and type can modulate SCFA absorption.

Summary

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

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Inflammation and Calcium Homeostasis: Potential Implications for the Transition Period

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Introduction

The vast majority of what we understand about calcium (Ca) homeostasis during the transition period was originally discovered by Dr. Ronald L. Horst (1949-2019) and his students/collaborators at the USDA National Animal Disease Center in Ames, IA. At lactation onset, dairy cows experience a marked increase (>65%; DeGaris and Lean, 2008) in Ca requirements to support colostrum and milk synthesis (Horst et al., 2005). The dairy industry has long hypothesized that the mammary gland's Ca demand is so extensive and acute that it often exceeds the homeostatic mechanisms (i.e., parathyroid hormone [PTH] and Vitamin D) employed to replenish it and as a result clinical or subclinical hypocalcemia (SCH) occurs (Horst et al., 2005; Goff, 2008). Implementing therapeutic and prophylactic strategies (i.e., pre-calving acidifying rations) has markedly reduced the incidence of clinical hypocalcemia (Charbonneau et al., 2006; Reinhardt et al., 2011), but SCH remains common. Some consider post-calving SCH as "pathological" and assume it causal in a myriad of seemingly unrelated negative health outcomes (ketosis, poor reproduction, displaced abomasum [DA], immune suppression, etc.; Caixeta et al., 2017; Rodríguez et al., 2017; Neves et al., 2018a,b). We suggest (outlined below) that periparturient immune activation contributes to clinical and SCH and the low circulating Ca is a consequence (a reflection) of inflammation rather than a predictor of future problems. In fact, we hypothesize that many post-calving undesirable phenotypes (reduced dry matter intake [DMI], hypocalcemia, elevated non-esterified fatty acids [NEFA], hyperketonemia) are a consequence of immune activation and not themselves causative of transition cow maladies.

Subclinical Hypocalcemia

Subclinical hypocalcemia remains a prevalent metabolic disorder afflicting ~25% of primiparous and ~50% of multiparous cows in the United States (Reinhardt et al., 2011). Although no overt symptoms accompany SCH, it has been loosely associated with poor gut motility, increased risk of DA, reduced production performance (i.e., milk yield and feed intake), increased susceptibility to infectious disease, impaired reproduction, and an overall higher culling risk (Curtis et al., 1983; Hansen et al., 2003; Seifi et al., 2011; Oetzel and Miller, 2012; Caixeta et al., 2017). Recent reports indicate that the severity of negative health outcomes observed in SCH cows appears dependent on the magnitude, persistency, and timing of SCH (Caixeta et al., 2017; McArt and Neves, 2019). For example, Caixeta et al. (2017) classified cases as either SCH or chronic SCH and observed more pronounced impairments on reproductive performance with chronic SCH. Similarly, McArt and Neves (2019) classified cows into 1 or 4 groups based on post-

calving Ca concentrations: normocalcemia (>2.15 mmol/L at 1 and 2 DIM), transient SCH (≤ 2.15 mmol/L at 1 DIM), persistent SCH (≤ 2.15 mmol/L at 1 and 2 DIM), or delayed SCH (> 2.15 mmol/L at 1 DIM and ≤ 2.15 mmol/L at 2 DIM). Cows experiencing transient SCH produced more milk and were no more likely to experience a negative health event when compared to normocalcemic cows, whereas the opposite (i.e., higher health risk and hindered productivity) was observed in cows experiencing either persistent or delayed SCH. Clearly not all cases of SCH are equivalent; in fact, transient hypocalcemia appears to be correlated with improved "health" and productivity and this may explain why inconsistencies exist in the relationship between SCH and reduced productivity and health (Martinez et al., 2012; Jawor et al., 2012; Gidd et al., 2015). However, it remains unclear why despite successful implementation of mitigation strategies, SCH remains prevalent, why SCH is associated with a myriad of seemingly unrelated disorders, and what underlying factors may be explaining the different "types" of SCH.

In addition to SCH, there are on-farm milk-fever situations that are biologically difficult to explain. For example, even while strictly adhering to a pre-calving calcium strategy, there remains a small percentage (~<1%) of cows that develop clinical hypocalcemia. Additionally, reasons for why a mid-lactation cow develops milk-fever are not obvious. Further, there appears to be an undecipherable seasonality component to clinical hypocalcemia in the southwest and western USA that coincides with the rainy season. Inarguably, there remain some aspects of Ca homeostasis that continue to evade discovery.

Inflammation in the Transition Period

Regardless of health status (Humblet et al., 2006), increased inflammatory biomarkers are observed in nearly all cows during the periparturient period (Ametaj et al., 2005; Humblet et al., 2006 Bionaz et al., 2007; Bertoni et al., 2008;; Mullins et al., 2012). The magnitude and persistency of the inflammatory response seems to be predictive of transition cow performance (Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018). During the weeks surrounding calving, cows are exposed to a myriad of stressors which may permit endotoxin entry into systemic circulation and thereby initiate an inflammatory response (Khafipour et al., 2009; Kvidera et al., 2017a; Proudfoot et al., 2018; Barragan et al., 2018; Koch et al., 2019). The frequency and severity of these inflammation-inducing insults presumably determines the level of inflammation that follows (Bertoni et al., 2008; Trevisi and Minuti, 2018). Common origins of endotoxin entry include the uterus (metritis) and mammary gland (mastitis). Additionally, we believe the gastrointestinal tract may contribute as many of the characteristic responses (rumen acidosis, decreased feed intake, and psychological stress) occurring during the transition period can compromise gut barrier function (see companion paper by Horst and Baumgard).

Although an overt inflammatory response is present around calving, numerous reports have described a reduction in immune competence during this time (Kehrli et al., 1989; Goff and Horst, 1997; Lacetera et al., 2005). Traditionally, hypocalcemia has been one of the primary factors considered responsible for periparturient immunosuppression

(Horst, 1997; Kimura et al., 2006), however, recent evidence suggests that the systemic inflammatory milieu may be mediating these effects (Heyland et al., 2006; Trevisi and Minuti, 2018). Furthermore, it was recently proposed that the immune system was not necessarily "suppressed," but merely dysregulated around calving (Trevisi and Minuti, 2018). Whether or not the immune incompetence frequently reported post-calving (Kehrli et al., 1989; Goff and Horst, 1997; Lacetera et al., 2005) is causative to future illnesses or is a consequence of prior immune stimulation needs further attention.

Inflammation and Metabolic Disorders

The periparturient period is associated with substantial metabolic changes involving normal homeorhetic adaptions to support milk production. Early lactation dairy cows enter a normal physiological state during which they are unable to consume enough nutrients to meet maintenance and milk production costs and typically enter into negative energy balance (NEB; Drackley, 1999; Baumgard et al., 2017). During NEB, cows mobilize NEFA in order to partition glucose for milk production in a homeorhetic strategy known as the "glucose sparing". Excessive NEFA mobilization and the affiliated increase in hepatic lipid uptake, triglyceride (TG) storage, and ketone body production has been traditionally believed to be the driving factor leading to ketosis and fatty liver (Grummer, 1993; Drackley, 1999). Until recently, this dogma has been well-accepted as ruminants are thought to have a poor capacity to export TG as very low density lipoproteins (Emery et al., 1992). However, increasing evidence suggests that chronic inflammation may be the driver of these disorders (Bertoni et al., 2006; Eckel and Ametaj, 2016) and this is supported by human, rodent, and ruminant literature which demonstrate effects of lipopolysaccharide (LPS) and inflammatory mediators on metabolism and hepatic lipid accumulation (Li et al., 2003; Barbuio et al., 2007; Endo et al., 2007; Bradford et al., 2009; Ilan et al., 2012; Ceccarelli et al., 2015). We and others have demonstrated that cows which develop ketosis and fatty liver postpartum had higher concentrations of LPS and acute phase proteins prior to diagnosis (Ohtsuka et al., 2001; Ametaj et al., 2005; Abuajamieh et al., 2016). Aside from the mechanistic changes on hepatic function, immune activation markedly reduces DMI (highly conserved response across species) which further increases NEFA mobilization and hepatic ketogenesis. Additional investigation is still needed to better elucidate the mechanisms by which LPS alters hepatic lipid handling and ketone synthesis and extra-hepatic ketone utilization.

Inflammation and Reproductive Function

Bacteria are ubiquitous within the postpartum uterus and pathogenic strains often persist leading to immune activation and consequently infertility (Sheldon and Dobson, 2004; Sheldon et al., 2019). Uterine infection has a variety of negative impacts on reproductive function including; a prolonged luteal phase (Peter and Bosu, 1988; Williams et al., 2008; Sheldon et al., 2009), disrupted ovarian steroidogenesis (Sheldon et al., 2009), and abnormal or delayed folliculogenesis after parturition (Huszenicza et al., 1999). Impaired reproductive functions are not isolated to infections which originate within the uterus. Mastitis, one of the most prevalent transition cow infections, disrupts follicular steroid concentrations and hinders oocyte maturation (Lavon et al., 2011; Asaf et al.,

2014). Furthermore, cows diagnosed with clinical mastitis prior to first service have an increased number of days to first service and days open (Barker et al., 1998). Administering LPS intravenously results in marked disruptions in hypothalamic and pituitary hormone release and ovarian responsiveness (Coleman et al., 1993; Battaglia et al., 2000) and induces abortion (Giri et al., 1990). Presumably, regardless of the origin, infection negatively influences immediate and future reproductive performance. The direct effects of endotoxin and inflammation on reproduction likely explain the associated effects that NEFA, ketones and calcium have with fertility (because immune activation also directly affects these metabolites; as described below).

Inflammation and Hypocalcemia

Impressively, immune activation was originally hypothesized by early investigators to be involved with milk-fever (Thomas, 1889; Hibbs, 1950), but until recently (Eckel and Ametaj, 2016) it has rarely been considered a contributing factor to hypocalcemia. Independent of the transition period, we and others have repeatedly observed a marked and unexplainable decrease in circulating calcium following LPS administration in lactating cows (Griel et al., 1975; Waldron et al., 2003; Kvidera et al., 2017b; Al-Qaisi et al., 2017; Horst et al., 2018a,b, 2019). Infection-induced hypocalcemia is a species conserved response occurring in humans (Cardenas-Rivero et al., 1989; Dias et al., 2013), calves (Tennant et al., 1973; Elsasser et al., 1996;), dogs (Holowaychuk et al., 2012), horses (Toribio et al., 2008), pigs (Carlstedt et al., 2000) and sheep (Naylor and Kronfeld, 1986). Additionally, hypocalcemia occurs in response to ruminal acidosis in dairy cows (Minuti et al., 2014). It is unlikely that cows (even those that are presumably "healthy") complete the transition period without experiencing at least one immune stimulating event and we are likely underestimating its contribution to postpartum hypocalcemia.

Traditional Dogmas

Long-standing tenets describe a causal role of hypocalcemia, increased NEFA, and hyperketonemia in the incidence of transition diseases and disorders (Figure 1). Hypocalcemia has traditionally been considered a gateway disorder leading to ketosis, mastitis, metritis, displaced abomasum, impaired reproduction, and decreased milk yield (Curtis et al., 1983; DeGaris and Lean, 2008; Goff, 2008; Martinez et al., 2012; Chapinal et al., 2012; Riberio et al., 2013; Neves et al., 2018a,b). The proposed mechanisms by which hypocalcemia leads to these ailments include impaired skeletal muscle strength and gastrointestinal motility (Goff, 2008; Oetzel, 2013; Miltenburg et al., 2016), decreased insulin secretion (Martinez et al., 2012, 2014), and the development of immunosuppression (Kimura et al., 2006). Similar to hypocalcemia, increased NEFA and hyperketonemia are presumed causative to illnesses such as DA, retained placenta, metritis, reduced lactation performance, poor reproduction, and an overall increased culling risk (Cameron et al., 1998; LeBlanc et al., 2005; Duffield et al., 2005; Quiroz-Rocha et al., 2009; Ospina et al., 2010; Chapinal et al., 2011; Huzzey et al., 2011). Additionally, elevated NEFA and ketones are thought to compromise immune function (Lacetera et al., 2004; Hammon et al., 2006; Scalia et al., 2006; Ster et al., 2012) and suppress feed intake

(Allen et al., 2009). Thus, the magnitude of changes in NEFA, BHB and Ca have traditionally thought to be predictors of future performance and problems.

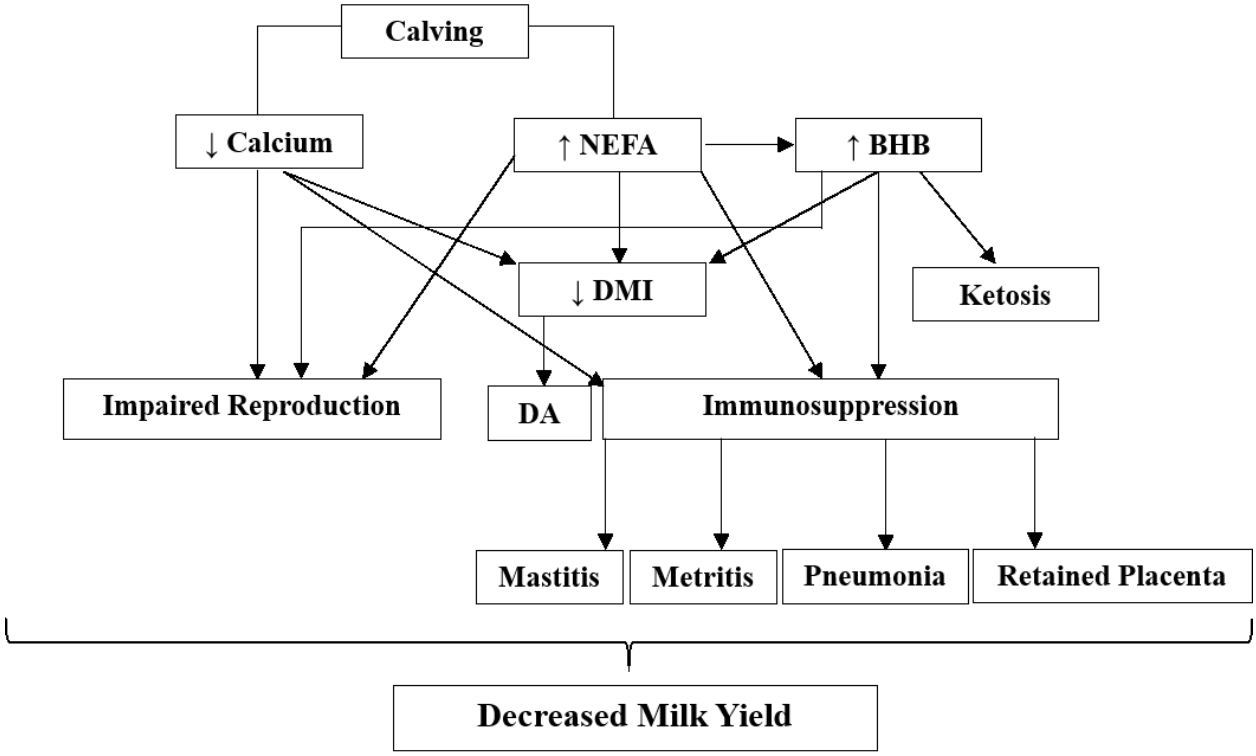

Figure 1. Traditional mechanisms by which hypocalcemia and increased NEFA and ketones are thought to cause poor transition cow health and performance.

Immune Activation: The Etiological Origin

Strong evidence has been generated connecting immune activation as the etiological origin of many of the metabolic and reproductive disorders traditionally observed within the transition period. Additionally, it is probable that immune activation is at least partially explaining the incidence of SCH in the postpartum period (Figure 2). It is intriguing to suggest that cases of delayed, persistent, and chronic SCH recently described by Caixeta et al. (2017) and McArt and Neves (2019) may be related to the severity of the periparturient inflammatory response. This hypothesis may explain why these cases of SCH are associated with reduced “health”, as these represent direct consequences of immune activation rather than being related or caused by decreased Ca. Regardless, these reports challenge the traditional dogmas surrounding hypocalcemia, elevated NEFA, and hyperketonemia and the sequence of transition cow disease.

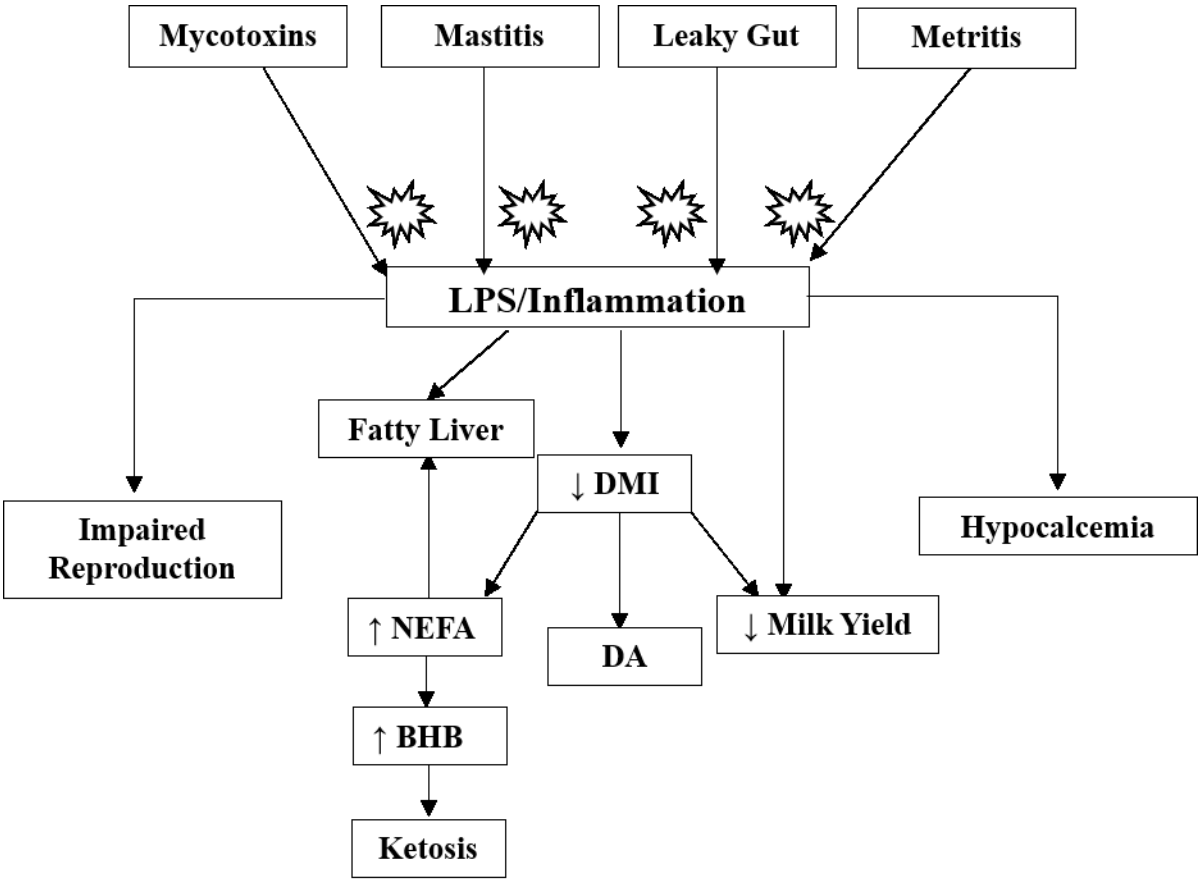

Figure 2. Potential downstream consequences of immune activation. In this model, decreased feed intake, hypocalcemia, excessive NEFA, hyperketonemia and hepatic lipidosis are not causative to poor transition cow performance and health, but rather a reflection of prior immune stimulation.

Calcium Administration following Immunoactivation

Although LPS-induced hypocalcemia is a commonly observed phenomenon, it remains poorly understood what role Ca plays in inflammation and why it acutely decreases. Recently, we investigated the effects of oral and i.v. Ca administration following an LPS challenge in lactating dairy cows (Al-Qaisi et al., 2017; Horst et al., 2018b). Administrating Ca (both orally and intravenously) successfully alleviated the magnitude of LPS-induced hypocalcemia (Figure 3A & B). Furthermore, utilizing a LPS-eucalcemic clamp technique we determined that the total Ca deficit was ~27 g during an acute (12 hour) and intense model of immune activation (Horst et al., 2018b).

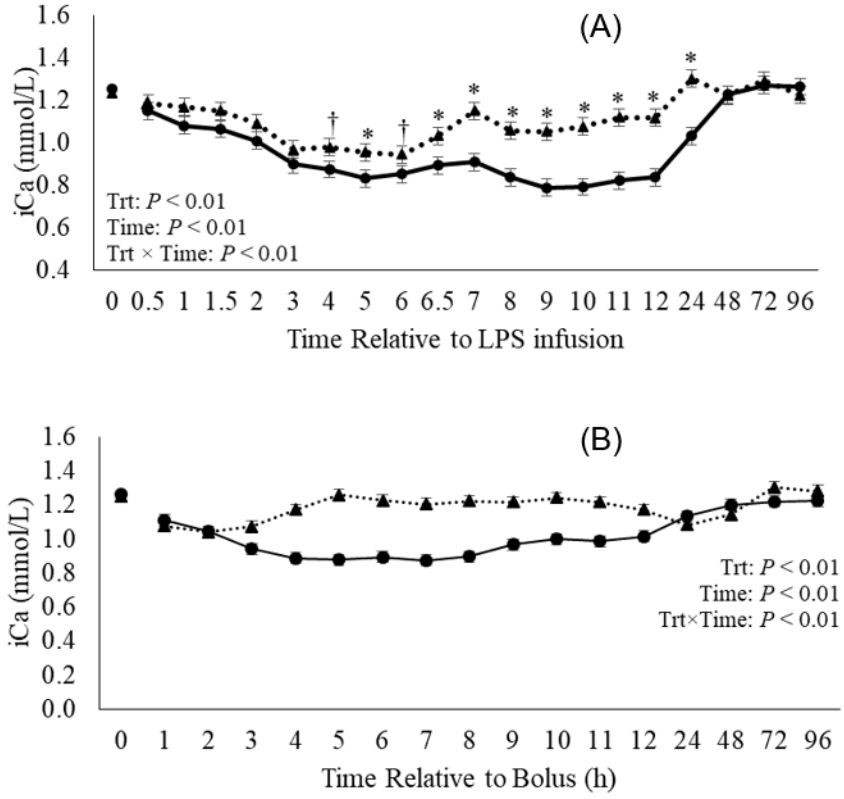

Figure 3. Ionized Ca concentrations from cows allowed to develop hypocalcemia (solid line) vs. cows administered oral (A) or intravenous (B) Ca (dashed line) following lipopolysaccharide infusion.

Although both models (oral and i.v. Ca) successfully lessened the degree of hypocalcemia, the impacts on productivity were markedly different. Administering oral Ca prior to and immediately following LPS administration improved milk yield and DMI when compared to cows allowed to become hypocalcemic (Al-Qaisi et al., 2017). In contrast, maintaining eucalcemia (via i.v. infusion) caused a more intense inflammatory response (i.e., increased acute phase proteins and rectal temperature) and impaired production performance (Horst et al., 2018b). Incidentally, LPS-induced severe hypocalcemia had no impact on neutrophil function nor did rescuing eucalcemia influence neutrophil function metrics (i.e., oxidative burst and myeloperoxidase activity; Horst et al., 2018b). Although it remains unclear why we observed conflicting results, it may be explained by the presence of both live yeast and Ca in the oral bolus. Yeast has previously been demonstrated to have immunomodulatory effects and benefit nutrient utilization, DMI, fermentation patterns, and lactation performance (Desnoyers et al., 2009; Broadway et al., 2015), therefore, we are unable to distinguish between the effects of live yeast vs. Ca in the oral bolus experiment. Another potential likely explanation for the conflicting results may be the route of administration. Intravenous Ca has recently been demonstrated to be detrimental to hormonal regulation of Ca when compared to oral boluses, and studies suggest it should not be utilized to treat subclinical cases (Wilms et al., 2019). Presumably, there are secondary signals associated with alimentary Ca absorption that

might explain why the oral Ca improved multiple productivity metrics following immunoactivation and the i.v. route did not.

Even though the results of the eucalcemic-clamp trial (Horst et al., 2018b) were surprising, they actually agree with the sepsis literature. Septic humans are typically hypocalcemic (Zaloga, 1992; Kelly and Levine, 2013) and early reports indicate that Ca administration to septic patients increased the incidence of organ failure and mortality (Malcolm et al., 1989). It is now hypothesized that sepsis-induced hypocalcemia serves as a protective strategy and should not be considered pathologic. Early investigators described a critical role of decreased blood Ca for optimal LPS detoxification via non-inflammatory routes (Figure 4; Skarnes and Chedid, 1964). In the absence of Ca, LPS aggregation is inhibited, a situation that allows LBP to transfer LPS monomers to cluster of differentiation 14 and eventually to acute-phase high density lipoproteins (ap-HDL) for biliary excretion. Formation of ap-HDL is mediated by SAA displacement of apolipoprotein from normal HDL (Skarnes and Chedid, 1964). This mechanism allows LPS to be detoxified with minimal leukocyte activation and thus less inflammation. In contrast, during eucalcemia the disaggregation of LPS monomers is inhibited (Skarnes and Chedid, 1964) and consequently, LPS is recognized by pro-inflammatory mechanisms; a scenario contributing to a hyper-inflammatory systemic response. Much remains unclear about why Ca decreases following immunoactivation, whether preventing it is beneficial, and where Ca is going during infection. These questions have direct implications to the periparturient inflamed dairy cow and to practical on-farm management and nutrition decisions.

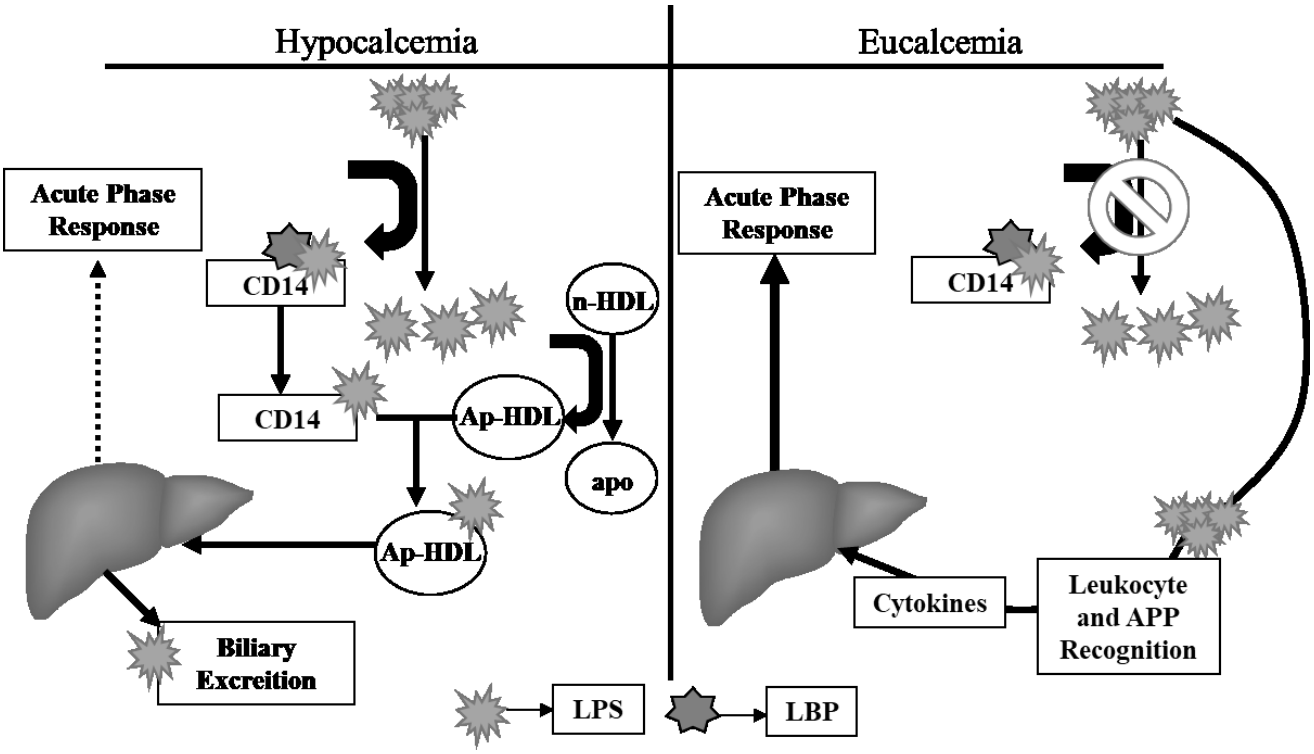

Figure 4. Calcium's proposed role in LPS detoxification.

Conclusion

Transient hypocalcemia remains a prevalent metabolic disorder afflicting dairy cows. Based upon the literature and our supporting evidence we suggest that SCH, along with the many disorders it is believed to be causal towards (i.e., ketosis, poor reproductive performance, metritis, etc.), can be explained (at least partially) by immune activation and the corresponding inflammatory response, a hypothesis which challenges several long standing dogmas in dairy science. More research is required to understand the mechanisms of infection induced-hypocalcemia and whether Ca administration (in particular the route of delivery) is beneficial or detrimental to SCH and future productivity. From a bigger picture perspective, we need a better understanding of whether or not these basic metabolites are “dangerous” (the reductionist theory) or are just reflective of prior immune insults.

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Herd Management Milk Analysis: Jersey versus Holstein and Between Lab Agreement of Results

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Introduction

In 2018 (Barbano et al., 2018), we provided a summary of how de novo, mixed, and preformed milk fatty acid concentrations in milk measured by mid-infrared (**MIR**) changed in relation to bulk tank fat and protein test for Holstein dairy herds. The analytical aspects of reference milk fatty acid (**FA**) analysis and model development and validation were reported by Wojciechowski and Barbano (2016) and Woolpert et al. (2016). The form of the FA data from the MIR (Barbano et al., 2014, 2017) was structured to provide information on the relative proportions of de novo (C4 to C15), mixed origin (C16:0, C16:1, C17:0), and preformed (C18:0 and longer) FA in milk, the mean FA chain length (carbon number) and degree of unsaturation (double bonds/fatty acid). Since that time, we have continued to collect data on milk FA variation in bulk tank milk for Jersey herds.

Woolpert et al. (2016, 2017) have reported the results of two studies to determine feeding and farm management factors influencing milk FA composition and their relationship to bulk tank milk fat and protein test and production per cow per day of fat and protein. In the first study (Woolpert et al., 2016) 44 commercial dairies were identified as either predominantly Holstein or Jersey in northern Vermont and New York. The yields of milk fat, true protein, and de novo FA per cow per day were higher for high de novo (**HDN**) versus low de novo (**LDN**) farms. Woolpert et al. (2016, 2017) estimated the impact of differences in de novo fatty acid concentration in milk among farms on bulk tank fat and protein, and estimated the impact of those differences on farm income per 100 cows per year. Higher milk de novo fatty concentration drove higher milk fat, milk protein, and grew revenues from milk. A study of Jersey herds from around the US was conducted during the past year to provide a comparison of milk fatty acid data mean data for a 16 month period for Holstein herds from the Northeast versus Jersey herds studied monthly for a 12 month period from different regions of the US. The relationship of milk fatty acid composition to bulk tank milk fat and protein test for both breeds of cattle is reported in this paper.

These relationships of milk fatty acid composition to bulk tank milk fat and true protein concentration are the basis of use of milk fatty acid composition in combination with herd management information to aid in making decisions to adjust dairy cattle ration composition or management to improve the production of milk fat, protein, and milk volume per cow per day. It has been shown that seasonal variation of fat and protein concentration in bulk tank milks in the northeastern US is related to seasonal varation in de novo fatty acid concentration and production in grams per cow per day (Barbano et al., 2017).

Our objective in the current work was to measure and compare the relationships of milk fatty acid composition and bulk tank fat and true protein test to milk fatty acid composition for Holstein versus Jersey farms. Based on data from our previous studies the following graphs (Figures 1,3,5,7,9 11, 13) for Holstein farms were developed to help farms understand the relationships between bulk tank milk FA composition and bulk tank milk fat and protein test. In the current paper, new data on bulk tank fat and protein tests and their relationship to milk fatty acid composition for bulk tank milk on Jersey farms (Figures 2, 4, 6, 8, 10, 12) are presented.

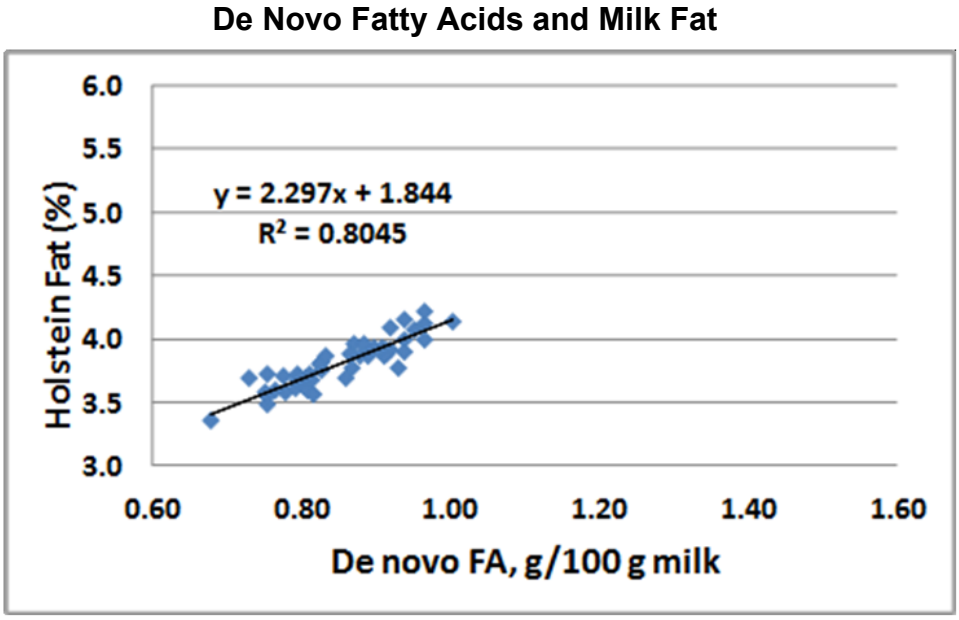

Figure 1. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of de novo FA in Holstein herd bulk tank milk.

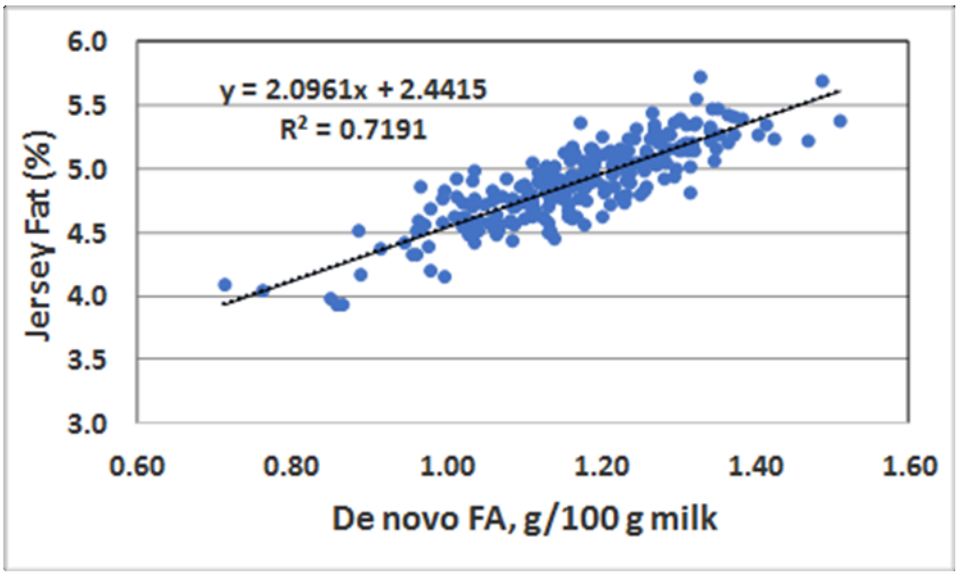

Figure 2. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of de novo FA in Jersey herd bulk tank milk.

Bulk tank milk fat concentration increases significantly ($P < 0.05$) with increasing de novo fatty acid concentration in milk for both Holstein and Jersey cattle with the slopes of these relationships being very similar.

Mixed Origin Fatty Acids and Milk Fat

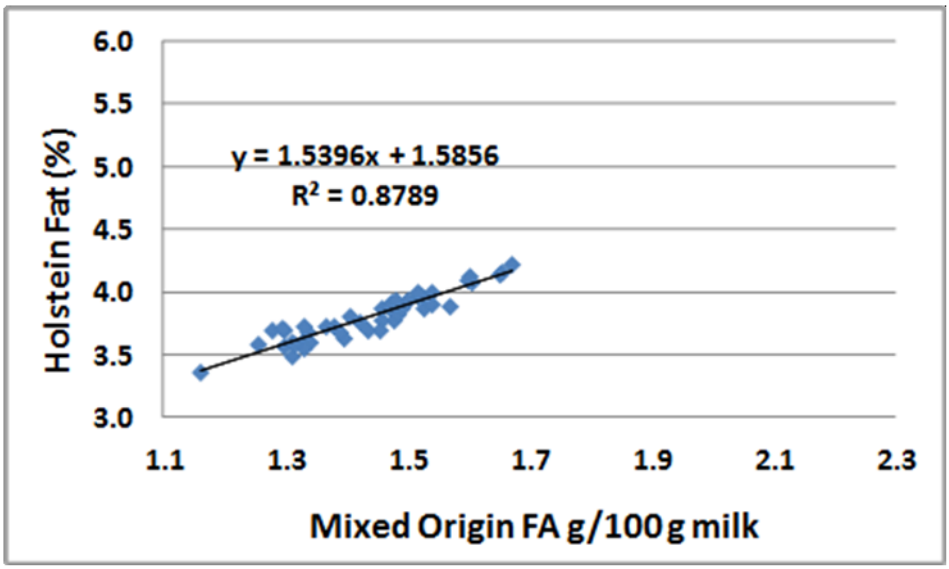

Figure 3. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of mixed origin FA in Holstein bulk tank milk.

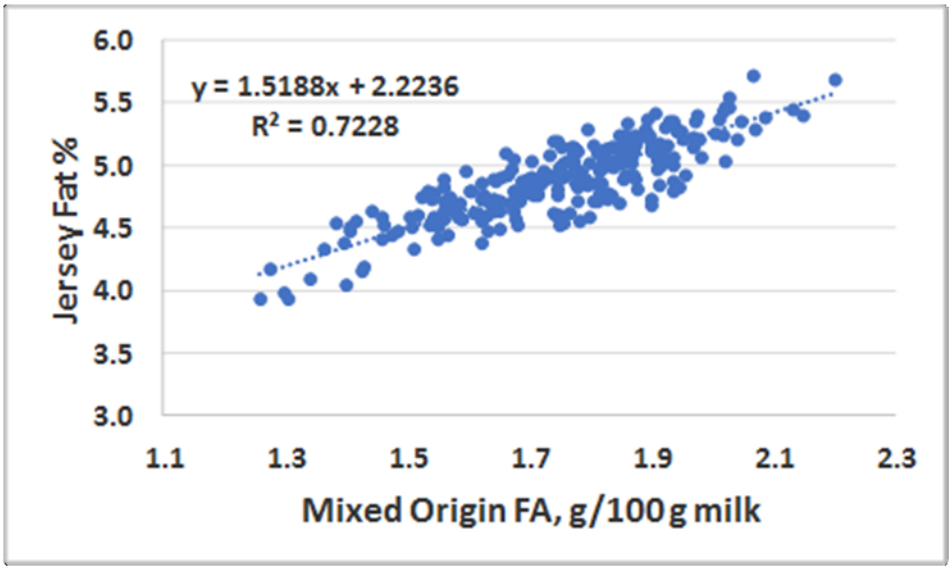

Figure 4. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of mixed origin FA in Jersey bulk tank milk.

Similar to what was observed for de novo fatty acids (Fgiures 1 and 2) bulk tank milk fat concentration increases significantly ($P < 0.05$) with increasing mixed origin fatty acid concentration in milk for both Holstein and Jersey cattle (Figures 3 and 4) with the slopes of these relationships being very similar.

De Novo plus Mixed Origin Fatty Acids and Milk Fat

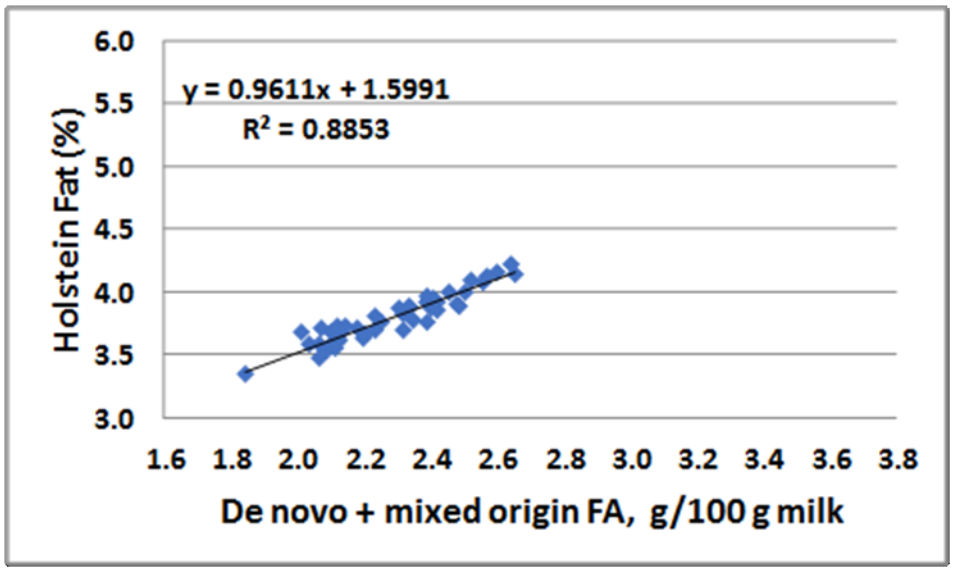

Figure 5. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of de novo plus mixed origin FA in Holstein bulk tank milk.

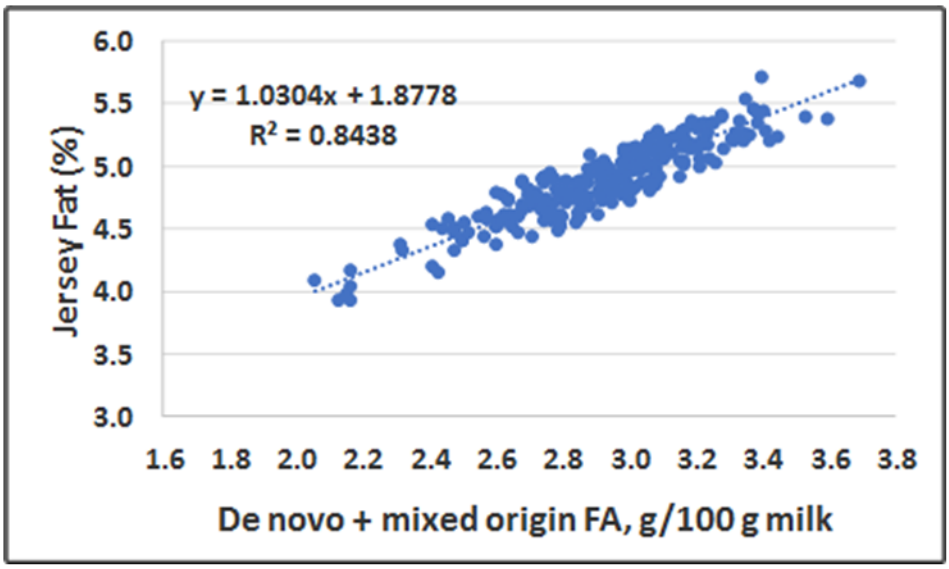

Figure 6. Relationship of bulk tank milk fat test to concentration (g/100 g milk) of de novo plus mixed origin FA in Jersey bulk tank milk.

Bulk tank milk fat concentration increases significantly ($P < 0.05$) with increasing de novo plus mixed origin fatty acid concentration in milk for both Holstein and Jersey cattle with the slopes of these relationships being very similar. The R^2 for these correlations are high

and consistent among the two breeds of cattle. On average a herd that does not have a seasonal calving pattern the average days in milk for the herd would be in the range of 150 to 200 days in milk. Therefore, on average the cows have a net positive energy balance and the bulk tank milk composition will be more strongly influenced by milk from cows in positive energy balance. With increasing days since calving the proportion of the palmitic acid (C16:0) in milk shifts palmitic acid originating from adipose tissue to palmitic acid be produced by the de novo synthesis pathway. Transfer of palmitic acid from by pass fat feeding when cow are in positive may impact this relationship and the ratio of mixed to de novo milk fatty acids.

Preformed Fatty Acids and Milk Fat

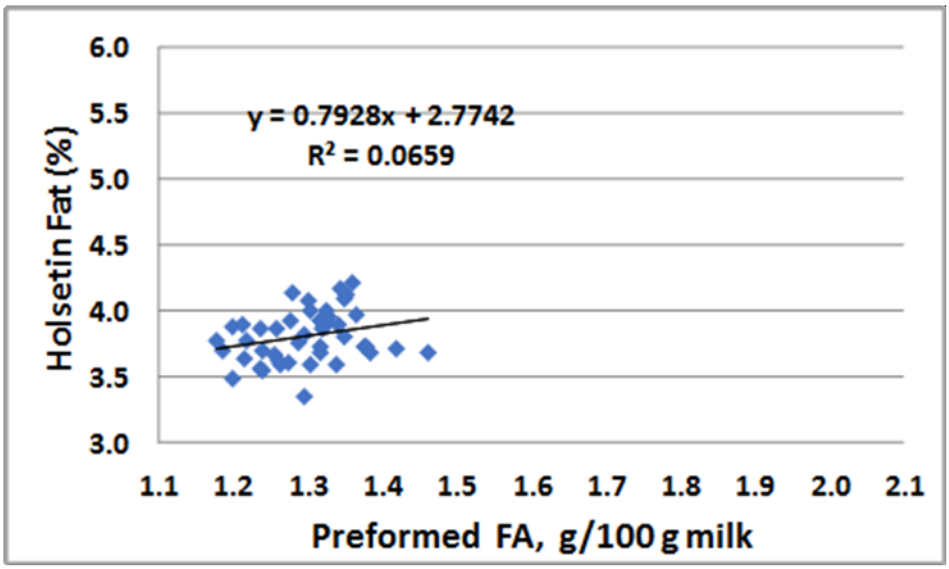

Figure 7. Relationship of Holstein bulk tank milk fat test to concentration (g/100 g milk) of preformed FA in bulk tank milk.

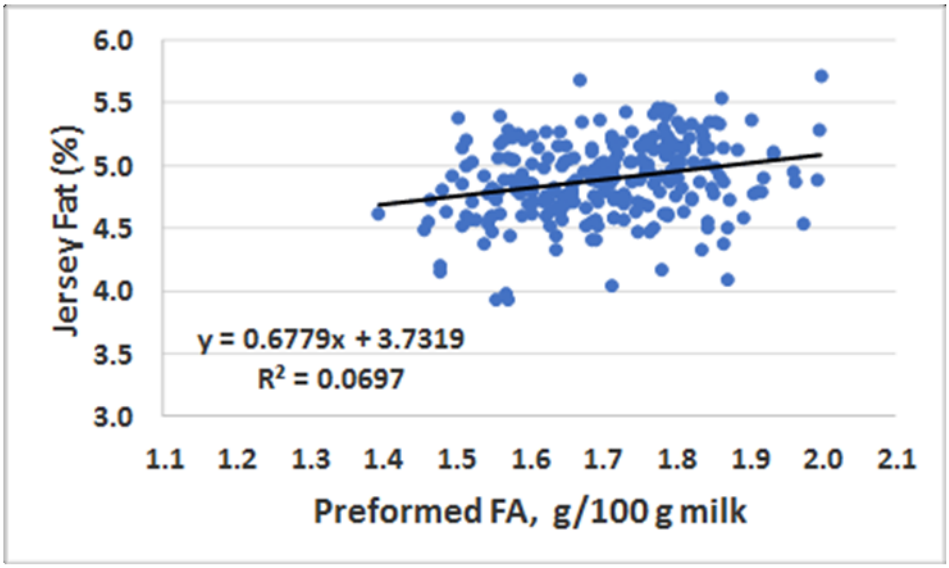

Figure 8. Relationship of Jersey bulk tank milk fat test to concentration (g/100 g milk) of preformed FA in bulk tank milk.

No increase in bulk tank milk fat concentration with increasing preformed fatty acid concentration in milk for both Holstein and Jersey cattle was detected ($P > 0.05$) with the slopes of these relationships not being significantly different from zero.

Double Bonds per Fatty Acid (Milk fat depression index) and Milk Fat

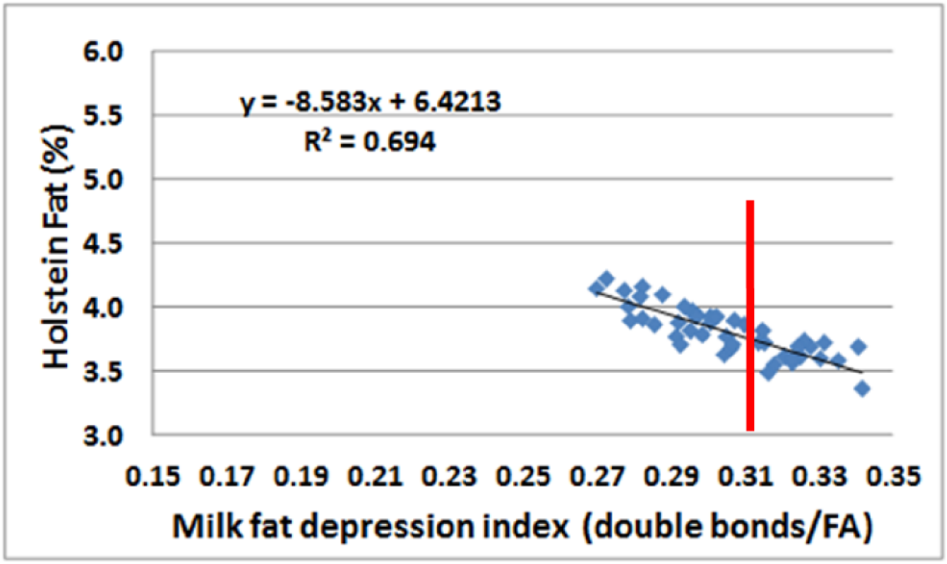

Figure 9. Milk fat depression index for Holstein bulk tank milk fatty acid unsaturation with bulk tank milk fat test. As double bonds per fatty acid increases the bulk tank milk fat test decreases. When double bond per fatty acid values are higher than the vertical line, there is a higher probability of unsaturated fat being too high or being released too fast in the ration.

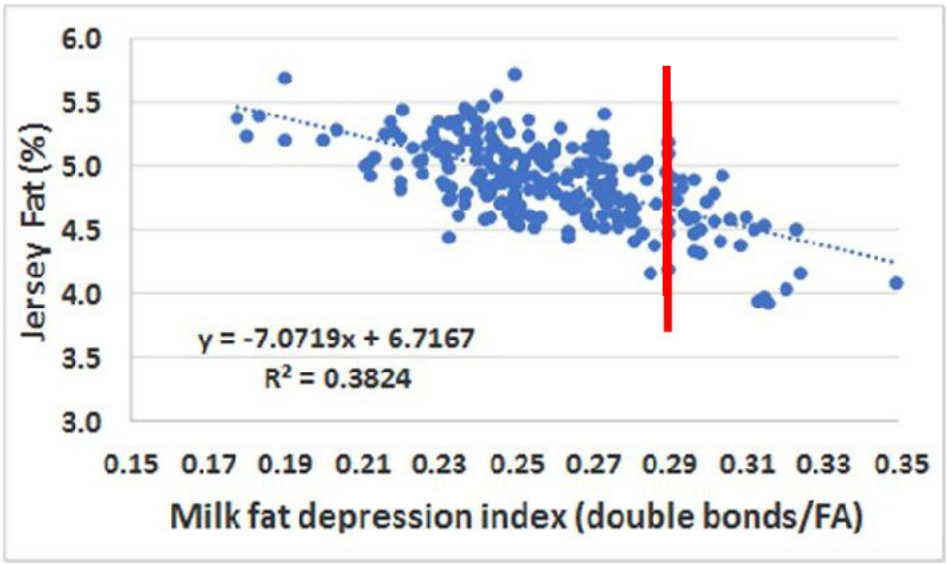

Figure 10. Milk fat depression index Jersey bulk tank milk fatty acid unsaturation with bulk tank milk fat test. When double bond per fatty acid values are higher than the vertical line, there is a higher probability of unsaturated fat being too high or being released too fast in the ration.

The PLS model for direct measurement of double bonds per fatty acid in milk fat was reported by Wojciechowski et (2016). As double bonds per fatty acid increases, the bulk tank milk fat test decreases. We have documented this in both Holstein and Jersey milks. In the Holstein milks represented in Figure 9, we have measured the level of C18:1 trans 10 fatty acid (with gas llquid chromatorgraphy) and in Holstein milks with a double bond per fatty acid higher than 0.31, the level of C18:1 trans 10 fatty acid is elevated indicating trans fatty acid induced milk fat depression. This is consistent with the report of Harvatine and Bauman (2011) that elevated levels of C18:1 trans 10, cis 12 CLA was related to milk fat depression. Similar results based on GLC analysis is found for Jersey cows, however the mean double bonds per fatty acid is lower for Jersey than for Holstein milk. Double bonds per fatty acid is an index and a high value for double bonds per fatty acid is an indicator of trans fatty acid induced milk fat depression. This is a valuble piece of farm management information when trying to interpret why overall milk fat percentage is low. If fat percent is low and the double bonds per fatty acid is low, then it is likely that the cause of the low fat is not classical trans fatty acid induced milk fat depression. Other causes of low milk fat may be low dry mater intake or other animal health issues that have caused immune system activation resulting in high demand for glucose to support the immune system response.

De Novo Fatty Acids and Milk Protein

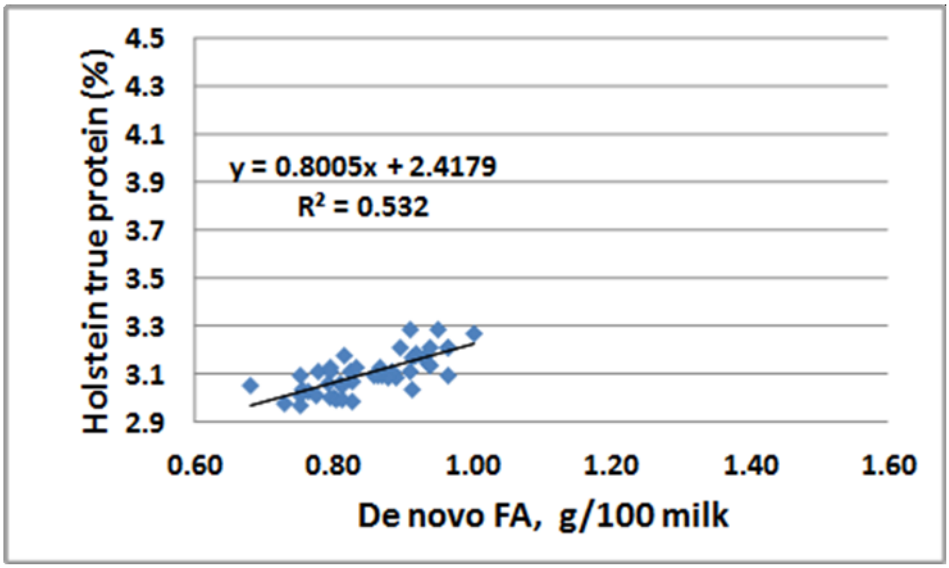

Figure 11. Relationship of Holstein bulk tank milk true protein concentration with change in de novo milk fatty acid concentration.

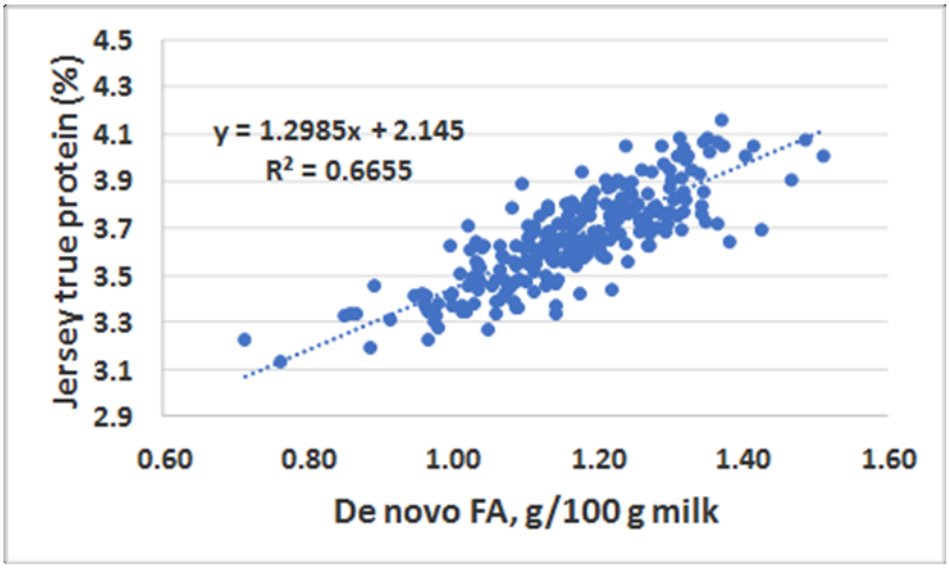

Figure 12. Relationship of Jersey bulk tank milk true protein concentration with change in de novo milk fatty acid concentration.

Milk true protein concentration increases with increasing milk de novo fatty acid concentration for both Holstein (Figure 11) and Jersey (Figure 12) bulk tank milks. Jersey have a larger increase in milk protein production per 100 grams of milk in response to increased denovo fatty acid production than Holsteins. Woolpert reported (2016, 2017) that dairy herds that had higher milk de novo fatty concentration produced more grams of protein per cow per day than herds that had low de novo fatty acid concentration in milk.

Preformed Fatty Acids and Milk Protein

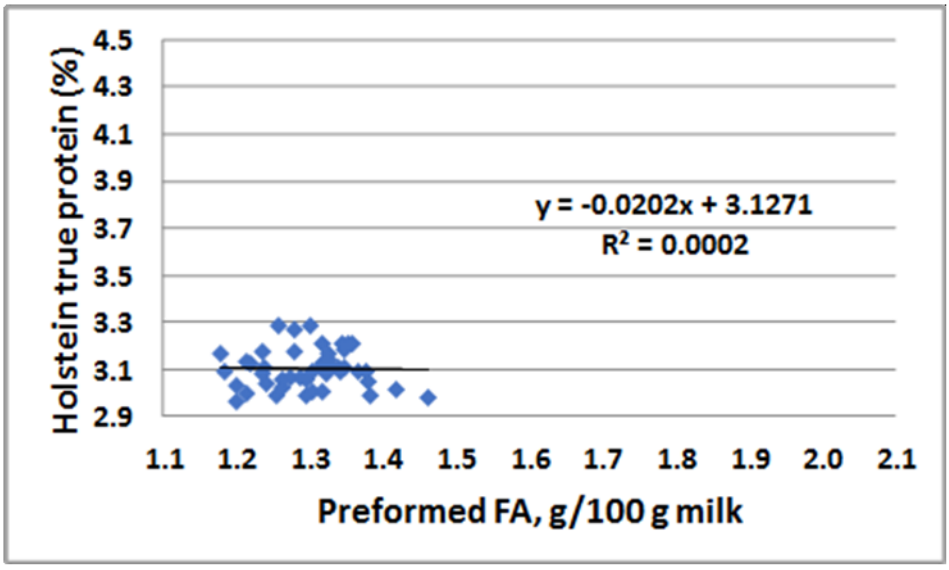

Figure 13. Relationship of Holstein bulk tank milk true protein concentration with change in preformed milk fatty acid concentration.

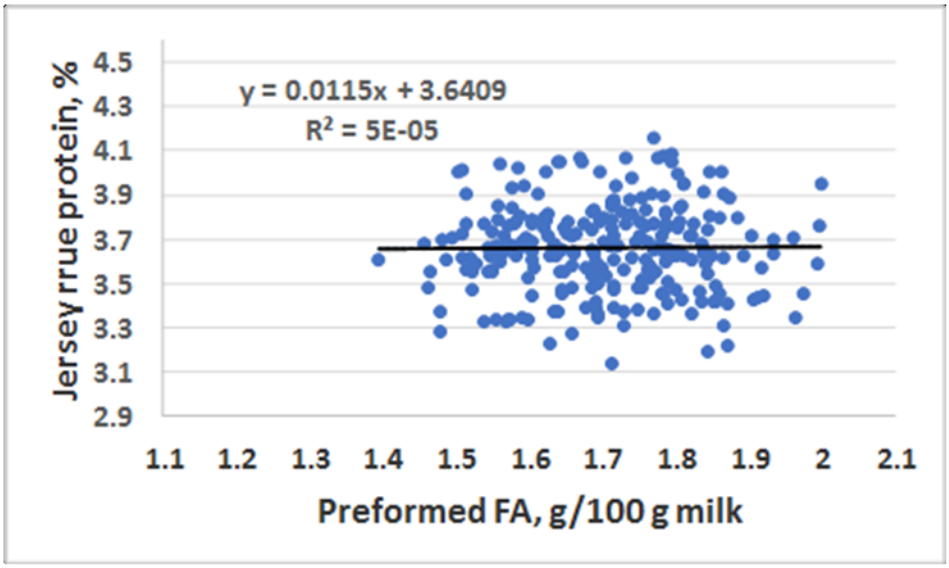

Figure 14. Relationship of Jersey bulk tank milk true protein concentration with change in preformed milk fatty acid concentration. As de novo milk fatty acid concentration increases milk protein increases.

As preformed milk fatty acid concentration increased no change in milk protein concentration was detected ($P > 0.05$) in either Holstein or Jersey bulk tank milk.

Between Lab Agreement Among Laboratories: Milk Fatty Acid Testing

Milk fatty acid prediction models need to be calibrated with reference milks that have reference values established by gas chromatography analysis (like calibration done for total fat, protein and lactose). The milk fatty acid GLC method calculation of results of GLC results for individual fatty acids, fatty acid chain length, and fatty acid double bonds per fatty acid used for this research was described by Wojceichowski et al. (2016) and Kaylegian et al. (2009 a,b). For this work only major fatty acids defined for the MIR application are included in the calculations and GLC data are normalized to 100% to ensure better lab-to-lab consistency of results of reference chemistry. This standardized approach will ensure better agreement among laboratories in both reference chemistry and MIR results.

Calibration samples for milk fatty acid analysis by MIR are produced at Cornell once every 4 weeks. The production of the calibration samples was described by Kaylegian et al., (2006a) and their performance for calibration of MIR milk analyzers for measuring milk fat, protein, lactose and solids was reported and compared to the use of individual farm milks (Kaylegian et al., 2006b). This same set of samples that is used across the US for calibration of MIR instruments for milk components is being used to calibrate MIR milk analyzers for milk fatty acid analysis with reference values expressed in grams per 100 gram of milk. The calibration set has a wide range of concentration of individual fatty acids and groups of fatty acids (e.g., de novo, mixed origin, and preformed fatty acids).

To validate the performance of a group of 9 MIR milk analyzers (Delta Instruments models FTA, combi 300, and combi 600 instruments), that were set up using the milk fatty

acid PLS models for direct measurement of fatty acid chain length and double bonds per fatty acid (Wojceichowski et al., 2016) and the PLS models for direct measurement of the groups of fatty acids defined as de novo, mixed origin, and preformed fatty acids reported by Woolpert et al. (2016). These instruments were calibrated (i.e., adjustment of secondary slope and intercept) for de novo, mixed orgin and performed fatty acid once every 4 weeks using the modified milk calibration samples (14 sample set) produced at Cornell University. The fatty acid chain length and double bonds per fatty acid models were calibrated periodically with a set of 8 individual producer milk samples with defined reference values. The instruments were calibrated approximately 2 weeks prior to the testing of the set of unknown validation samples. The validation samples were 8 individual farm milks. There were 2 farm milks from each of 4 different geographic regions of the US. This set of validation milks was tested by gas chromatography to establish reference values and was tested on each of 9 different MIR milk analyzers in different regions of the US.

A typical example of the reference chemistry for set of modified milk calibration samples for milk fatty acids is shown below in Table 1.

Table 1. Modified milk calibration sample reference chemistry.

Sample	total grams de novo fatty acid (g/100g milk)	total grams mixed origin fatty acid (g/100g milk)	total grams preformed fatty acid (g/100g milk)	total grams fat (g/100g milk)
1	0.0471	0.0846	0.0731	0.2167
2	0.1400	0.2514	0.2174	0.6438
3	0.2347	0.4215	0.3644	1.0793
4	0.3291	0.5912	0.5111	1.5139
5	0.4231	0.7600	0.6570	1.9461
6	0.5150	0.9250	0.7997	2.3688
7	0.6091	1.0941	0.9459	2.8017
8	0.7028	1.2624	1.0914	3.2326
9	0.7968	1.4311	1.2373	3.6648
10	0.8896	1.5979	1.3814	4.0918
11	0.9844	1.7681	1.5287	4.5278
12	1.0767	1.9340	1.6721	4.9526
13	1.1722	2.1055	1.8204	5.3918
14	1.2629	2.2685	1.9612	5.8090
Mean	0.6560	1.1782	1.0187	3.0172
min	0.0471	0.0846	0.0731	0.2167
max	1.2629	2.2685	1.9612	5.8090
Range	1.2158	2.1839	1.8881	5.5924

The results of the MIR milk analysis of the validation samples is given in Tables 2 through 6 below.

Table 2. Reference values and individual laboratory predictions for de novo fatty acid concentration (g/100 g milk) in 8 individual farms milk validation samples and calculated mean difference (MD) and standard deviation of the difference (SDD) from the reference values fore each of 9 different laboratories running Delta FTA or Delta Combi MIR milk analyzers.

	denovo		Lab	Lab	Lab	Lab	Lab	Lab	Lab	Lab	lab
Sample	Reference		1	2	3	4	5	6	7	8	9
1	0.8991		0.860	0.862	0.874	0.860	0.870	0.894	0.920	0.890	0.890
2	0.8484		0.820	0.810	0.838	0.820	0.822	0.828	0.840	0.820	0.830
3	0.7209		0.720	0.732	0.743	0.730	0.715	0.748	0.750	0.720	0.720
4	0.8179		0.810	0.811	0.819	0.800	0.789	0.804	0.840	0.800	0.830
5	0.7540		0.720	0.729	0.754	0.750	0.731	0.740	0.740	0.730	0.740
6	0.9635		0.930	0.937	0.964	0.940	0.933	0.953	0.950	0.930	0.950
7	0.7910		0.810	0.798	0.803	0.820	0.796	0.804	0.840	0.810	0.810
8	1.3033		1.220	1.224	1.252	1.240	1.234	1.220	1.240	1.230	1.250
	0.8873	Mean	0.861	0.863	0.881	0.870	0.861	0.874	0.890	0.866	0.878
		MD	-0.026	-0.024	-0.006	-0.017	-0.026	-0.013	0.003	-0.021	-0.010
		SDD	0.031	0.029	0.023	0.029	0.022	0.032	0.035	0.027	0.022

The agreement of mean values among MIR instruments for de novo fatty acids was excellent and they were in good agreement with the GLC reference chemistry for these samples.

Table 3. Reference values and individual laboratory predictions for mixed origin fatty acid concentration (g/100 g milk) in 8 individual farms milk validation samples and calculated mean difference (MD) and standard deviation of the difference (SDD) from the reference values fore each of 9 different laboratories running Delta FTA or Delta Combi MIR milk analyzers.

	Mixed		Lab	Lab	Lab	Lab	Lab	Lab	Lab	Lab	lab
	Reference		1	2	3	4	5	6	7	8	9
1	1.3295		1.480	1.445	1.438	1.420	1.419	1.471	1.480	1.490	1.460
2	1.1070		1.220	1.170	1.162	1.180	1.163	1.168	1.170	1.220	1.200
3	0.9481		1.050	1.042	1.041	1.010	0.996	1.035	1.030	1.060	1.040
4	1.1063		1.240	1.232	1.208	1.210	1.158	1.186	1.260	1.260	1.230
5	1.0260		1.100	1.098	1.103	1.100	1.049	1.078	1.070	1.100	1.080
6	1.3599		1.490	1.455	1.472	1.440	1.414	1.482	1.440	1.450	1.460
7	1.3105		1.330	1.261	1.267	1.300	1.227	1.225	1.290	1.300	1.280
8	1.5220		1.660	1.625	1.648	1.640	1.580	1.630	1.650	1.680	1.620
Mean	1.2136		1.321	1.291	1.292	1.288	1.251	1.285	1.299	1.320	1.296
		MD	0.108	0.077	0.079	0.074	0.037	0.071	0.085	0.106	0.083
		SDD	0.043	0.055	0.054	0.039	0.052	0.070	0.059	0.057	0.051

Overall, the between lab agreement of MIR instruments for mixed orgin fatty acids was good but the mean estimate by the group of instruments was a bit higher (0.08 g/100 g of milk) than GLC reference chemistry on this group of validation samples.

Table 4. Reference values and individual laboratory predictions for preformed fatty acid concentration (g/100 g milk) in 8 individual farms milk validation samples and calculated mean difference (MD) and standard deviation of the difference (SDD) from the reference values for each of 9 different laboratories running Delta FTA or Delta Combi MIR milk analyzers.

	Preformed		Lab	Lab	Lab	Lab	Lab	Lab	Lab	Lab	lab
	Reference		1	2	3	4	5	6	7	8	9
1	1.4988		1.370	1.419	1.426	1.480	1.451	1.405	1.410	1.380	1.390
2	1.4982		1.390	1.479	1.492	1.450	1.468	1.484	1.470	1.400	1.440
3	1.5371		1.410	1.438	1.427	1.460	1.480	1.458	1.470	1.390	1.490
4	1.5798		1.440	1.471	1.544	1.510	1.561	1.563	1.430	1.400	1.490
5	1.4224		1.370	1.371	1.370	1.380	1.438	1.429	1.440	1.350	1.460
6	1.7128		1.560	1.635	1.606	1.690	1.677	1.622	1.660	1.620	1.660
7	1.3716		1.310	1.414	1.434	1.370	1.442	1.477	1.410	1.340	1.400
8	1.7819		1.690	1.739	1.695	1.750	1.784	1.774	1.730	1.650	1.760
Mean	1.5503		1.443	1.496	1.499	1.511	1.538	1.526	1.503	1.441	1.511
		MD	-0.108	-0.055	-0.051	-0.039	-0.013	-0.024	-0.048	-0.109	-0.039
		SDD	0.036	0.049	0.058	0.026	0.041	0.066	0.059	0.046	0.052

Overall, the between lab agreement of MIR instruments for preformed fatty acids was good but the mean estimate by the group of instruments was a bit lower (0.054 g/100 g of milk) than GLC reference chemistry on this group of validation samples. It has been our experience on prediction of mixed and preformed milk fatty acids with the first generation of PLS prediction models on the Delta instruments, that when the models for mixed origin and preformed milk fatty acids do not agree with GLC reference chemistry they are off in opposite directions. A second generations of fatty acid PLS models is under development.

Table 5. Reference values and individual laboratory predictions for mean fatty acid chain length (carbon number) in 8 individual farms milk validation samples and calculated mean difference (MD) and standard deviation of the difference (SDD) from the reference values for each of 9 different laboratories running Delta FTA or Delta Combi MIR milk analyzers.

	CL		Lab	Lab	Lab	Lab	Lab	Lab	Lab	Lab	Lab
Sample	Reference		1	2	3	4	5	6	7	8	9
1	14.7434		14.63	14.76	14.80	14.72	14.65	14.65	14.67	14.76	14.76
2	14.7429		14.64	14.78	14.79	14.69	14.61	14.69	14.71	14.77	14.78
3	14.8803		14.75	14.85	14.91	14.83	14.76	14.73	14.82	14.88	14.88
4	14.7634		14.64	14.72	14.76	14.68	14.64	14.65	14.64	14.77	14.73
5	14.7897		14.67	14.75	14.78	14.71	14.66	14.67	14.73	14.76	14.78
6	14.8062		14.61	14.74	14.77	14.69	14.63	14.61	14.70	14.77	14.77
7	14.7861		14.67	14.79	14.83	14.73	14.68	14.69	14.73	14.76	14.82
8	14.4498		14.32	14.38	14.46	14.37	14.25	14.32	14.32	14.43	14.47
Mean	14.7452		14.616	14.721	14.763	14.678	14.610	14.626	14.665	14.738	14.749
		MD	-0.129	-0.024	0.017	-0.068	-0.135	-0.119	-0.080	-0.008	0.004
		SDD	0.029	0.039	0.032	0.028	0.037	0.043	0.035	0.023	0.028

The 9 instruments in the validation study had not had a calibration adjustment for milk fatty acid chain length (Table 5) or double bonds per fatty acid (Table 6) in 8 months. It has been our experience that the calibration PLS models for prediction of structural parameters such as fatty acid chain length and double bonds per fatty acid are much more stable across time (versus concentration parameters). The agreement of among instruments for mean fatty acid chain length was good with the mean value about 0.06 carbons lower than the reference value for this set of validation samples.

Table 6. Reference values and individual laboratory predictions for mean fatty acid unsaturation (double bonds per fatty acid) in 8 individual farms milk validation samples and calculated mean difference (MD) and standard deviation of the difference (SDD) from the reference values for each of 9 different laboratories running Delta FTA or Delta Combi MIR milk analyzers.

	DB/FA		Lab	Lab	Lab	Lab	Lab	Lab	Lab	Lab	Lab
Sample	Reference		1	2	3	4	5	6	7	8	9
1	0.2651		0.260	0.275	0.289	0.270	0.281	0.277	0.260	0.290	0.270
2	0.2974		0.290	0.308	0.318	0.288	0.301	0.310	0.300	0.310	0.300
3	0.3405		0.320	0.329	0.344	0.326	0.334	0.328	0.330	0.340	0.340
4	0.2987		0.290	0.299	0.311	0.291	0.307	0.309	0.290	0.310	0.300
5	0.3237		0.310	0.316	0.325	0.305	0.319	0.321	0.310	0.320	0.320
6	0.3065		0.290	0.299	0.310	0.286	0.301	0.293	0.300	0.310	0.300
7	0.2841		0.280	0.302	0.311	0.282	0.302	0.306	0.290	0.300	0.300
8	0.2649		0.250	0.255	0.273	0.245	0.259	0.268	0.250	0.260	0.250
Mean	0.2976		0.286	0.298	0.310	0.287	0.301	0.302	0.291	0.305	0.298
		MD	-0.011	0.000	0.013	-0.011	0.003	0.004	-0.006	0.007	0.000
		SDD	0.006	0.011	0.010	0.009	0.010	0.013	0.007	0.010	0.009

The agreement of among instruments for mean fatty acid unsaturation was excellent both from instrument to instrument and in agreement with reference chemistry for this set of validation samples.

Take Away Messages

1) The relationships documented among milk fatty acid composition and bulk tank milk fat and true protein test in Jersey milk follows the same relationships that have been documented for Holstein dairy herds.

2) Jersey cows produce more milk fat and tend to have a higher relative concentration of de novo and mixed origin fatty acids than observed for Holstein cows.

3) Jersey cows seem to have a larger increase in milk true protein concentration than Holstein cows as milk de novo fatty concentration increases.

4) Jersey cows have a shorter mean milk fatty acid chain length and lower mean unsaturation than milk from Holstein cows. However, the relationship of decreasing fat and protein concentration in milk with increasing mean unsaturation is similar in both breeds and higher level of mean double bonds per fatty acid is an indicator of trans fatty acid induced milk fat depression.

5) Between lab agreement between the MIR compared in this study milk fatty acid analysis was very good for instruments that were calibrated every 4 weeks with reference. The between laboratory agreement was best for de novo fatty acid content (g/100 g milk) and milk fat depression index (double bonds per fatty acid).

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Effect of forage inclusion in diets on milk yield and milk components of dairy crossbreed ewes managed on an accelerated lambing system

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Introduction

Sheep dairies produce 1.4% of the global milk for human consumption. Sheep milk is rarely consumed as fluid milk. Instead, it is mostly processed into cheeses, whey cheeses and yogurt (Bencini, 2002). Dairy sheep production is heavily concentrated in the Mediterranean, with France, Greece, and Italy, as well as Romania, being leading producers (Balthazar et al., 2017).

Sheep flocks are managed to lamb and start lactating within a short period of time between early fall and late spring in Europe (Sitzia et al., 2015) and mostly in early spring in the United States (Jaeggi et al., 2005), with one annual lactation (~160 days) (Table 1). Most sheep milk is seasonally available. While seasonal production systems are warranted in mountainous regions and Mediterranean Europe, sustainable sheep milk production in the US Northeast – with abundant forage and achievable cost-effective production of high-quality winter feed – is important for economical farm viability.

Table 1. Milk production of relevant breeds in the US and North America.

	Breed				
Item	East Friesian	Lacaune	Finnsheep	Dorset	Finnsheep × Dorset
Reference	(Thomas, 2014)	(Thomas, 2014)	(Sakul and Boylan, 1992)	(Sakul and Boylan, 1992)	(Kochendoerfer and Thonney, 2018)
Region	US	US	North America	North America	Cornell sheep flock
Lactation yield, kg	209.4	194.8	66.0	72.0	176.3[a]
Lactation days	161	155	~120[2]	~120[b]	122[a]
Fat, %	5.8	6.3	6.0	6.5	6.3
Protein, %	4.8	5.2	5.4	6.1	5.3
Lactose, %			4.7	4.8	4.8
Milk solids, %			16.8	18.2	

[a]1.45 kg/day on average with 73 days lactation length, 1.67 times per year.
[b]Milking started in late April or early May and lasted until the first week of September.

Between 2016 and 2018, a flock of 46 meat-breed Finnsheep × Dorset cross ewes were milked on the STAR-accelerated lambing system to achieve year-round sheep milk production. With 1.67 lambings per year, the ewes achieved 176.3 kg of milk per year. Litter size and conception rates were very high with 3.5 lambs per ewe sold or retained as replacements per year and 87% conception rates across the 9 breeding seasons of the 2-year experiment. Aseasonally polyestrous meat sheep breeds can be utilized for milk production in accelerated lambing systems in short (73-day) lactations and can be managed for meat and milk production with year-round product availability.

However, farmer and stakeholder commentary from the US Northeastern sheep dairying community suggested that 5 lambing periods per year are too labor intensive to be manageable in a small-scale, on-farm, setting. Additionally, the pelleted research diet did not reflect actual Northeastern sheep dairy diets due to low forage inclusion (300 g hay per head per day).

A feeding and milking trial was designed to test the following hypotheses: 1) Crossbreeding with East Friesian dairy sheep genetics will achieve extended lactation persistency to 120 days and thus allow for a step down in lambing intensity from the STAR accelerated lambing system, 2) Crossbreeding will retain out-of-season breeding ability of the hybrid ewes and achieve an accelerated lambing system with 1.5 lambings per ewe per year and 3 lambing periods per year, and 3) Higher forage inclusion will negatively affect milk yield.

Methods

In February 2019, 23 Finnsheep × Dorset × ¼ East Friesian first parity ewes that were 11 to 12 months of age at lambing, were randomly assigned to 1 of 2 pens within 2 dietary groups, HF (High Forage) and LF (Low Forage). Both groups were offered the same completely balanced, pelleted concentrate diet ad libitum plus hay (Table 2). The HF group was offered hay ad libitum, while hay intake was restricted for the LF group. These diets were fed for about 10 days prior to lambing. Lambs were removed 12 hours after birth and reared artificially on cold milk offered ad libitum. After removal of the lambs, the dams were milked in a low-line, 6-stanchion parlor, at the Cornell University Teaching Barn twice daily at 7 am and 5 pm. Feed intake (pen values) was recorded daily. Milk yield and samples for NIRS component analysis (Woolpert, 2017) were collected from each ewe once per week. Ewes and lambs were weighed weekly. Blood samples for metabolite analyses were collected prepartum, and on days 1, 7, and 40 of lactation. Feed and fecal samples were collected for digestibility analysis (Thonney, 1979).

Quadratic equations were fitted to milk protein and fat yield as well as component concentrations. An exponential equation was fitted to the milk yield data [1]. Total lactation yield was calculated by integrating this equation [2], peak yield [3] and day of peak [4] were calculated using the first derivative of the equation (Wood, 1967):

[1] $$Y = ax^b \exp(-cx)$$

[2] $$Y = \frac{\mathrm{a}}{\mathrm{c}^{\mathrm{b}+1}} \Gamma(b+1)$$

[3] $$Y_{(max)} = a\left(\frac{b}{c}\right)^b \exp(-b)$$

[4] $$X = \frac{b}{c}$$

The lactation yield and component curve parameters were analyzed statistically for effect of diet with a linear model including fixed effects of diet and pen within diet and with maximum days in milk as continuous covariate using *lm* in R (R-Core-Team, 2019).

Table 2. Composition of experimental diets (% of DM).

Ingredient	35% pfNDF[a]	2nd cutting hay
Soy hulls	42.4	
Wheat midds	20.1	
Corn	24.1	
Soybean meal	8.6	
Molasses	1.7	
Cornell sheep premix	1.06	
Ammonium chloride	0.78	
Calcium carbonate	1.12	
Pellet binder	0.26	
Measured components		
DM (% of feed)	89.5	90.9
DDM (Dairy One estimated TDN)	80.6	59.0
CP	17.0	14.2
NDF	41.1	54.4
pfNDF	35.1	26.4
INDF	6.0	28.0
NSCHO	34.0	24.4
EE	2.6	5.0
Ash	5.3	5.0

[a]Pelleted diet, offered ad libitum to both groups.

Serum NEFA concentrations were determined enzymatically [ELISA] using the HR series NEFA-HR (2) Wako kit (Wako Life Sciences, Inc. Mountain View, CA) according to the manufacturer's instructions. Samples were plated and run in triplicate. The data were analyzed as a mixed linear model with the *lme4* package in R (Bates et al., 2015) with effects of diet, pen within diet, ewe as a random variable, and the 4 sampling timepoints and their interactions with diet as fixed effects.

Feed intake data are expressed as raw means in percentages of body weight.

Results and Discussion

Ewes in both groups consumed on average 3.1 kg DM per day, with ewes in the LF group consuming 2.73 kg DM of the concentrate diet and 0.37 kg hay, and the HF group consuming less concentrate (2.60 kg) and more hay (0.50 kg) daily. With average body weights of 72.6 kg for the HF group and 70.8 kg for the LF group, the ewes consumed 4.3% and 4.4% of their BW, respectively. Thus, feed intake was high with lower forage inclusion resulting in slightly higher feed intake.

The estimates of the lactation curve parameters a, b, and c were slightly different but not statistically different between the two forage inclusion levels (Table 3). Daily milk yield and total lactation yield were higher ($p < 0.04$) for the LF diet group. Peak lactation was substantially higher ($p = 0.011$) in the LF group, with peaks occurring earlier than for the HF group. Milk protein and fat percentages were higher for the HF group, but protein and fat lactation yield overall was higher for the LF dietary treatment. This reflects the differences between forage levels in milk yield (Table 3)**Error! Reference source not found.**.

Table 3. Statistical analysis.

	Diet			
	HF	LF	SEM	p-value
a	2.29	2.21	0.329	ns
b	-0.02	0.10	0.085	ns
c	0.004	0.009	0.0026	ns
Daily milk yield, kg	1.55	1.96	0.134	0.042
Total lactation yield, kg	166	213	14.3	0.033
$DIM_{(max)}$	110	105		
Peak yield, kg	1.67	2.70	0.245	0.011
Day of peak	27	16	5.07	ns
Protein %	5.6	5.0	0.83	ns
Total milk protein yield, kg	8.6	9.3	0.86	ns
Fat %	6.1	5.3	0.84	ns
Total milk fat yield, kg	9.4	10.3	0.68	ns

The raw milk yields are plotted in Figure 1 together with the average of fitted lactation curves for the HF and LF levels. Reflected by the variation in milk production among ewes, none of the ewes had been selected previously for milk production. Due to the small size of the Cornell sheep flock and the even smaller size of the East Friesian crossbreed dairy flock, selecting for milk production through culling low producing animals has not yet been an option. Additionally, literature investigating Italian Sarda ewes suggested that high variation is common in ewe milk production (Cappio-Borlino et al., 1995). Further investigation into the shape of lactation curves for meat × dairy crossbreed ewes is necessary to find the best ways to describe lactation curves capturing the high early peak yields often found for traditionally meat breed ewes (Reynolds and Brown, 1991). Future breeding and selection for milk production will achieve higher lactation yields.

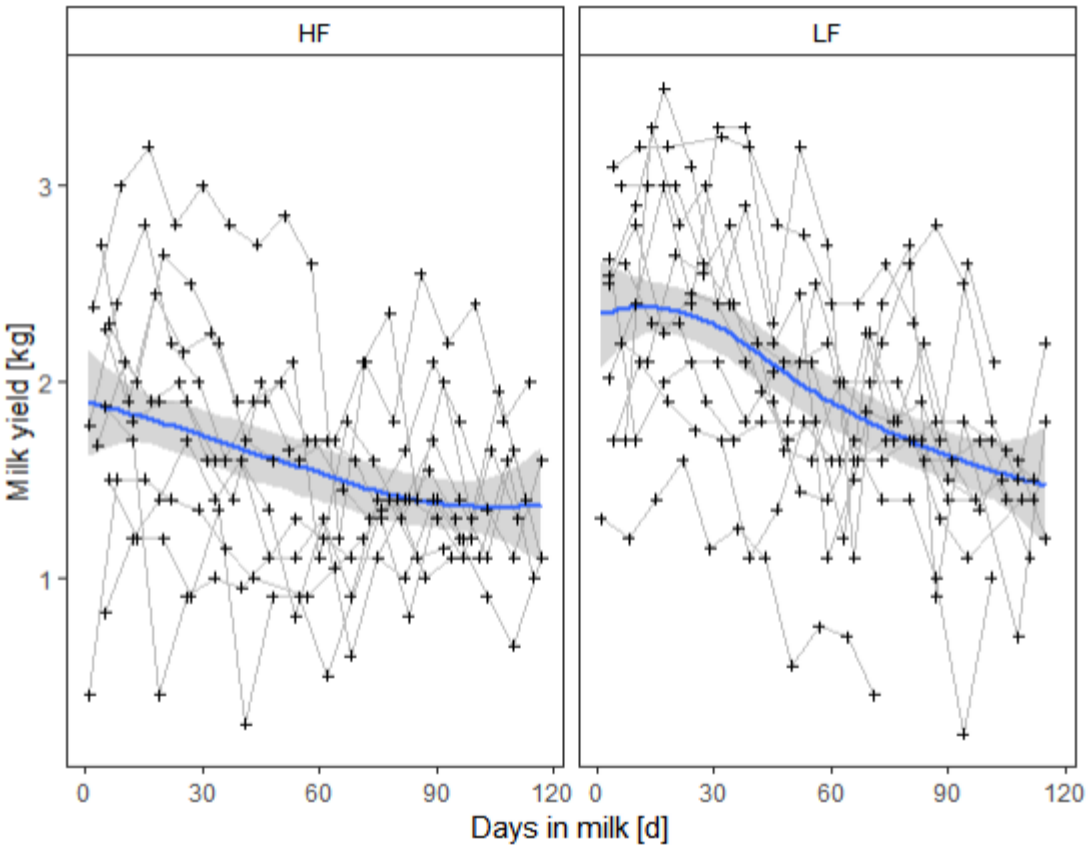

Figure 1. Lactation curves for HF dietary treatment (left) and LF dietary treatment (right). Raw data plotted with regression line and 95% CI.

Litter size differences between dietary groups were small, with 1.3 and 1.5 lambs born for the HF and LF groups, respectively. There was no effect of litter size on milk yield. The literature suggests smaller litter sizes for US East Friesian dairy sheep (Thomas, 2014) than for Finnsheep × Dorset cross breeds (Kochendoerfer and Thonney, 2018). The lower litter sizes for ewes in this experiment may be due to ewes being in their first parity.

There was no effect of dietary forage level on serum NEFA levels of ewes in early lactation. Differences in digestible nutrient intake between the diets may not have been large enough to affect serum NEFA concentrations. Overall, the serum NEFA concentrations were very low (< 300 μeq/L), indicating that ewes were in positive nutrient balance and were not mobilizing adipose tissue reserves.

With ewes being re-bred on day 97 of their lactation utilizing teaser rams between 17 and 10 days prior to breeding and CIDRs between 7 and 0 days prior to breeding (Inskeep, 2011) and with a conception rate of 82%, we can conclude that the out-of-season breeding ability of the Finnsheep × Dorset crossbreed ewes was retained in our ¼ East Friesian yearlings.

Summary

Similar to our previous milking experiment utilizing aseasonally polyestrous meat breeds, this experiment was successful in achieving high milk yields. With a total lactation yield of 213 kg for the LF group in 105 days of lactation, milk yields were higher than previously reported lactation yields for East Friesian dairy ewes (Thomas, 2014). Considering that the accelerated management system has 1.5 lactations per year, yearly lactation yield will be even higher. This experiment was designed as a cross-over experiment with the same group of ewes starting to lamb and lactate again in their second parity at the beginning of October 2019. Ewes previously enrolled in the HF group will receive the LF inclusion diet and vice versa. Further, feed chemistry as well as protein requirements for wool growth will be determined, analyzed, and put into context. Fatty acid composition of the milk will be illuminating in terms of mammary uptake of dietary, long-chain fatty acids and short-chain de novo fatty acid production within the mammary gland and potential effects of diet on milk fat composition.

Preliminarily we conclude that 1) crossbreeding with East Friesian dairy breed genetics may lead to extended lactation persistency compared with meat breed sheep; 2) the ability to conceive out-of-season is retained in these hybrid ewes; and 3) higher forage inclusion (< 200 g additional voluntary forage intake) leads to lower daily and lactation yields with no effect on milk components.

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Ruminal Acidosis: Beyond pH and Rumen

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Introduction

Studies show that acidosis is a very significant disorder of cattle. Studies in Wisconsin found that 20.1 and 23% of cows had subacute acidosis as defined by rumen pH <5.5 (Oetzel et al., 1999; Oetzel, 2004) and others in Ireland had 11% (O'Grady et al., 2008). A large Australian study found that 10% of dairy cows <100 days in milk had acidosis, as defined by assessment of ruminal VFA, ammonia, lactic acid, and pH (Bramley et al., 2008). Therefore, it is likely that many cows will experience some level of acidosis during lactation and, indeed, some may be affected many times. It can be estimated that if the prevalence of sub-acute acidosis is 10% (Bramley et al., 2008) and the duration of a case is 2 days based on data by Golder et al. (2014b), then there would be an incidence of approximately 1500 cases over a 300 d lactation per 100 cows. Understanding and controlling acidosis is therefore critical to ensuring animal well-being and production.

There is now considerable debate about the definition of acidosis with papers providing varying definitions, many based on ruminal pH, others referring to conditions not solely based on ruminal changes (Plaizier et al., 2018) and some based on a series of different rumen measures (Bramley et al., 2008; Golder et al., 2014; Lean et al., 2013; Morgante et al., 2007). Providing thoroughly defensible definitions of the condition is critical to management of acidosis, because a failure to properly define the condition in scientific experiments can lead to a failure to adequately control the condition. In this paper, we discuss definitions of acidosis, provide some suggestions for definitions and examine recent findings on rumen function that may help prevent acidosis. Lastly, we evaluate evidence that there is cross-talk between the mammal and bacteria and these interactions influence the outcomes of rumen function.

What is acidosis?

Researchers, primarily based in the EU, state that ‘The classification of and terminology used in relation to dietary-induced disorders of the ruminant digestive system are confused and not fit for purpose. The problem is most apparent in relation to the condition referred to as sub-acute rumen acidosis (**SARA**), for which there are no adequate, accepted criteria for definition. Sub-acute is a poorly defined descriptor of the time-course of a disease and is often misinterpreted to refer to either subclinical disease or disease in which clinical signs are mild.’ We agree with their synopsis and provide the following supported thoughts to provide definitions of these conditions that may help with diagnosis and prevention of the disorder.

Acidosis is a continuum of conditions of varying severity that reflect the challenge of safely sequestering hydrogen that accumulates from carbohydrate fermentation. Safe pools to 'hide hydrogen' include starch engulfment by protozoa, bacterial glycogen formation, growth of bacteria, methane, and weak organic acids (VFA). Less safe pools include lactic acid, because that acid is 10 times stronger than the VFA. Decreasing the hydrogen supply by increasing the more slowly fermenting fiber content of the diet and enhancing rumination can reduce the risk of acidosis. It is important to recognize that the effects, and possibly even pathogenesis of acidosis may not be solely ruminal and other parts of the gastro-intestinal tract play a role.

Acute Acidosis

Acute acidosis is defined by the generation of significant amounts of lactic acid in the rumen. Nagaraja and Titgemeyer (2007) characterize acute acidosis as being present when rumen pH is <5.0, there is >50 mM lactic acid and ruminal VFA are less than 100 mM. Other studies support these criteria (Golder et al., 2014a; Golder et al., 2014b). There is a general consistency of definition and understanding of this condition in the literature. Acute acidosis is caused by the sudden access of cattle to rapidly fermentable carbohydrates (**RFCHO**) or changed processing of the same RFCHO. Fructose appears to have greater potential to cause acute acidosis than starches (Golder et al., 2012b; Golder et al., 2014b) and glucose has been used to create lactic acidosis (Nagaraja et al., 1981). Acute acidosis is characterized by fatal or serious disorder.

Definition

Acute acidosis is a serious condition of cattle characterized by death, dehydration, ruminal distension, diarrhea (often with grain in the feces and a sickly, sweet smell), abdominal pain, tachycardia, tachypnea, staggering, recumbency, coma, a marked decline in milk yield, and sequalae including ruminitis, liver abscess, pulmonary infections, epistaxis, and poor production that arises subsequent to the ingestion of large amounts of RFCHO. The rumen fluid can be milky white often containing grain and has a pH of <5.0, >50 mM lactic acid, and VFA <100 mM.

Acidosis

The definition 'sub-acute' does not sit easily in definitions that apply to metabolic diseases. It is simpler and more correct to ignore the term 'sub-acute'. Lean et al. (2009) provided a series of conditions that define metabolic disease based on the postulates of Evans (1976). It is clear that increasing dietary starch (Li et al., 2012), sugars (Nagaraja et al., 1981; Golder et al., 2012b), changing the forage fed (Khafipour et al., 2009), and changing the particle size of the feed (Zebeli et al., 2012), can create acidosis and meet the postulates proposed (Lean et al., 2009). However, there is very considerable variation in the responses of individual cattle to the increase in RFCHO and rumen pH is not the most consistent and easily measured change in rumen outcomes.

Plaizier et al. (2018) highlight a large number of studies that estimate the prevalence of low rumen pH, but cows with low pH did not have significantly different clinical outcomes to other cows, apart from low body condition score. By way of contrast, Bramley et al. (2008) who used both rumenocentesis and stomach tube measures of ruminal pH, but also ruminal VFA and ammonia concentrations found that the rumen pH measures were not highly predictive for a group of cows that were characterized by being in herds where dietary NFC were higher, NDF lower, and that had a markedly (>100%) higher incidence of lameness (Bramley et al., 2013) than other herds. The best predictors for these cows that also had a low milk fat to milk protein content and ratio, was a combination of rumen VFA concentrations, particularly valerate and propionate and rumen ammonia. The least predictive, albeit significant, variables for classifying cows as acidotic were rumen pH and lactic acid. In this paper, we explore the implications of these findings and support for them. Further, it is important to recognize that there is the potential for hindgut changes to influence outcomes of a RFCHO challenge (Gressley et al., 2011).

We consider that the following factors, some of which we explore in this paper are likely to influence the expression of acidosis i) production of toxic substances and clearance of these from the rumen. The generation of toxins and clearance of toxins will be influenced by ruminal populations of micro-organisms; ii) compromised epithelia, through chemical action, conditions such as pestivirus that damage epithelial integrity and ability to appropriately process toxins, iii) rate of passage and differential clearance and exposure of different parts of the gastrointestinal tract. All of the above functions may be influenced by genetics and understanding the interactions of these with the metabolome (physiological responses) and metataxome (the population of rumen microbes) is an important new frontier. Consequently, we propose the following definition.

Definition

Acidosis is a serious condition of cattle characterized by cyclic inappetence, increased risk of lameness, diarrhea (often with grain and/or gas in the feces), increased risk of low milk fat percentage, and sequalae including ruminitis, liver abscess, pulmonary infections, abomasal displacement, epistaxis, and poor production that arises subsequent to the ingestion of large or moderate amounts of rapidly fermentable carbohydrates.

On a herd basis, findings include: variable individual production, high prevalence (>40%) of lameness (Bramley et al., 2013), high prevalence of milk fat to milk protein ratio <1.02, and diets that are high in NFC >40%, but low in NDF <31%. Findings based on Bramley et al. (2008) include rumen fluid that is high in total VFA > 100 mM, of moderately low pH (<5.8 rumenocentesis or 6.2 stomach tube), with concentrations of propionate >30 mM and low ammonia <3 mM.

Other observations likely to be pertinent to increasing the risk of acidosis include evidence of sorting of diets, overstocking of corrals, mixing of heifers and cows and mixing of new cattle (Lean et al., 2014).

Limitations of pH as a diagnostic measure

The series of changes caused by the increase in RFCHO extends well beyond a decrease in pH and includes changes in a vast number of metabolic pathways involved in acidosis including the generation of potentially toxic metabolites (Ametaj et al., 2010a; Zhang et al., 2017). Zhang et al. (2017) found ruminal increases in amino acids, bacterial degradation products including amines, and sugars with increased concentrates fed. There is considerable speculation in regards to the agents that might be implicated in causing some of the clinical signs of acidosis and Lean et al. (2013b) summarized some of the evidence supporting potential roles for histamine, endotoxin, and lactic acid to cause laminitis (Table 1). Given, the known agents capable of causing inflammation and clinical signs, and that less well-known metabolites may be involved in clinical signs of acidosis, it is unsurprising that rumen pH *per se* is largely unrelated to the clinical signs of acidosis. Given the large number of potential toxins, often produced simultaneously in the rumen (Ametaj et al., 2010), a singular focus on any particular toxin is not appropriate.

Table 1. Summary of the evidence supporting the potential for histamine, endotoxin and lactic acid to cause laminitis on diets rich in rapidly fermentable carbohydrates. Sourced from Lean et al. (2013b)

	Histamine	Endotoxin	Lactic acid
Generated in the rumen	√	√	√
Absorbed by healthy rumen	√	√[a]	√
Absorbed by damaged rumen	√	?	?[b]
Induced laminitis when injected	√	√	√

[a]Evidence is inconsistent [b]Appears to be probable

More critically perhaps, in terms of diagnostic potential, a highly accurate measurement of rumen pH is nearly impossible. Simply, the rumen is dynamic and not homogenous and any measure whether continuous and indwelling, or static, regional and singular has limitations. Similar observations can be made in regard to most rumen measures, as rumen function varies within the rumen mat, liquid phase, and near the rumen wall and papillae (Penner, 2014). Table 2 from Golder (2014) shows the differences and correlations between different measures of rumen pH. Figure 1 derived from Bramley et al. (2008) shows the correlations between rumen samples drawn by stomach tube and rumenocentesis in 660 cows (R^2 = 0.2). Table 3 shows the value of different tests for acidosis and highlights that rumen pH provided very similar results whether obtained by stomach tube or rumenocentesis. An extensive series of studies in the United Kingdom with indwelling pH meters demonstrated that these could detect changes in the diet of cattle, but variability in individuals in their baseline pH and responses to diet did not provide adequate diagnostic outcomes for predicting differences among individual cattle without careful use of complex statistics (Denwood et al., 2018). It is, however, this large variability among cattle that provides the most interesting directions for research and prevention of acidosis in the future.

Table 2. Difference and relationship between ruminal pH measurements in ruminal fluid collected using stomach tubing, rumenocentesis, and rumen fistula methods in cattle

Methods compared	No. of cows sampled	Difference in ruminal pH values between methods[1]	Relationship between methods (r^2)	Reference
Stomach tube and rumenocentesis				
	6	+0.04		Shen et al. (2012)
	58	+0.76	0.11	Enemark et al. (2004)
	5	+1.1		Nordlund et al. (1995)
	660	+0.54	0.20	Bramley unpublished
	16	+0.35	0.25	Duffield et al. (2004)
Rumenocentesis and fistula				
	30	+0.28	0.52	Garrett et al. (1999)
	16	+0.33	0.42	Duffield et al. (2004)
	30	+0.34	0.73	Garrett et al. (1995)
Stomach tube and fistula				
	16	+0.34	0.58	Duffield et al. (2004)
Continuous ruminal pH measurement system and fistula			Correlation coefficient (r)	
Mean over 1 min	14	Mean of 1 and 5 min	0.98	Penner et al. (2006)
		-0.03		
Mean over 5 min	14		0.97	Penner et al. (2006)
	4	-0.04	0.99	Sato et al. (2012)
	4	+0.39	0.93	Phillips et al. (2010)
	12	+0.11	0.85	Dado and Allen (1993)
	6		0.65	Graf et al. (2005)
	1	-0.07	0.88	AlZahal et al. (2007)
	16	cranial-ventral site	0.68	Duffield et al. (2004)
	16	caudal-ventral site	0.61	Duffield et al. (2004)
	16	central site	0.35	Duffield et al. (2004)
	16	cranial-dorsal site	0.50	Duffield et al. (2004)
Continuous ruminal pH measurement system and stomach tube				
	16	First sample	0.15	Duffield et al. (2004)
	16	Second sample	0.31	Duffield et al. (2004)
Continuous ruminal pH measurement system and rumenocentesis				
	16		0.43	Duffield et al. (2004)
	6		0.56	Marchesini et al. (2013)

[1]Difference in ruminal pH values were calculated by subtracting the mean ruminal pH value for the second named ruminal collection method from the first named collection method ie Mean ruminal pH of stomach tube ruminal sample - Mean ruminal pH of rumenocentesis ruminal sample.

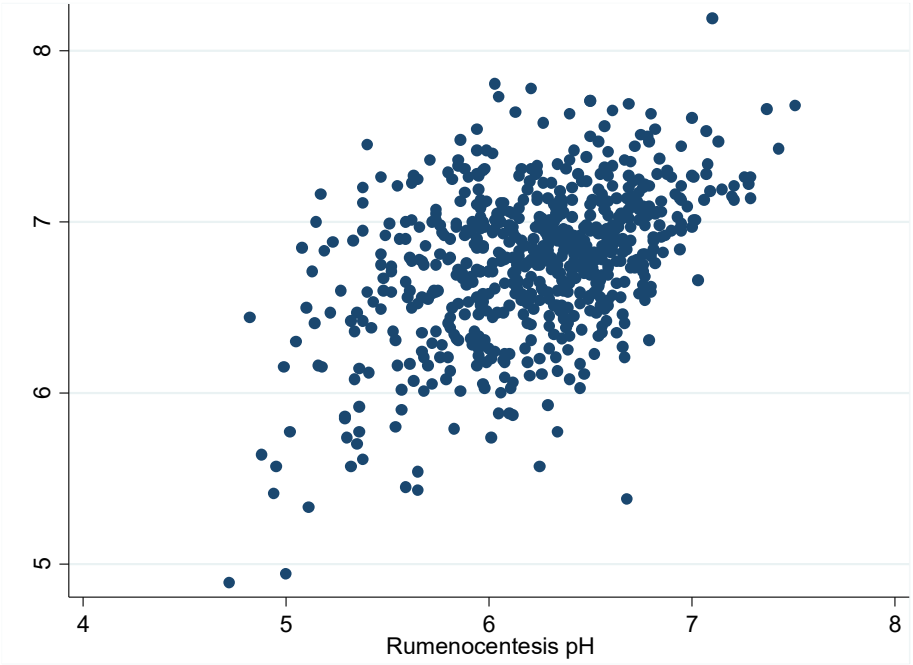

Figure 1. Scatter plot comparing rumen pH measured by rumenocentesis vs. stomach tube (R^2 = 0.20) Sourced from Bramley et al. (2008)

Table 3. Sensitivity, specificity, area under the curve, and cut-off points from receiver operator curves for the acidosis diagnostic value of rumen and milk measure from samples obtained by Bramley et al. (2008). Sourced from Golder et al. (2012a)

Measure	Sensitivity	Specificity	Area under the curve	Cut-points
Acetate (mM)	0.94	0.27	0.627	36.7
Butyrate (mM)	0.94	0.20	0.530	5.28
Propionate (mM)	0.93	0.87	0.955	23.10
Valerate (mM)	0.90	0.90	0.954	1.62
pH (Stomach tube)	0.68	0.84	0.801	6.54
pH (Rumenocentesis)	0.74	0.79	0.822	5.96
Milk Fat:Protein	0.54	0.81	0.716	1.02

Is there a good test for acidosis?

For a test to be effective, it needs to be able to be both sensitive ie detect true cases of the condition and be specific, that is have few false positive detections and be applicable across a wide range of conditions. Bramley et al. (2008) conducted their study on a wide range of herds that fed only pasture, through to different levels of grain and supplement feeding including total mixed ration herds. Herd was not a significant factor in the study in the prediction of acidosis. Subsequent, tightly controlled challenge studies using 1.2% of BW fed as grain, showed that propionate, ammonia, and valerate concentrations were the most sensitive indicators of the potential for different grains to cause acidosis (Lean et al., 2013a), and that the Bramley model was sensitive to ruminal change consistent with acidosis.

Further, a study performed using gradated steps of 2 kg of additional supplement, primarily wheat grain, but also canola meal demonstrated that as supplement increased, so did acidosis as measured using the Bramley model and that at 16 kg of supplement all cattle were acidotic most of the day (Figure 2). The cattle with acidosis had decreased milk production and milk fat percentage; however, feeding the supplement as a part of a mixed ration or substituting some of the wheat for canola deceased the prevalence of acidosis. There were very few cattle with acidosis in the low supplement groups and a high prevalence in the high supplement groups. It appears that the model for evaluating acidosis is fit for purpose, but requires a method to simply apply in the field. While it is likely that this model will be refined, the critical value in the model is that it demonstrates that acidosis is much more than pH and that performance of cattle is much more closely aligned to a model that considers more than pH.

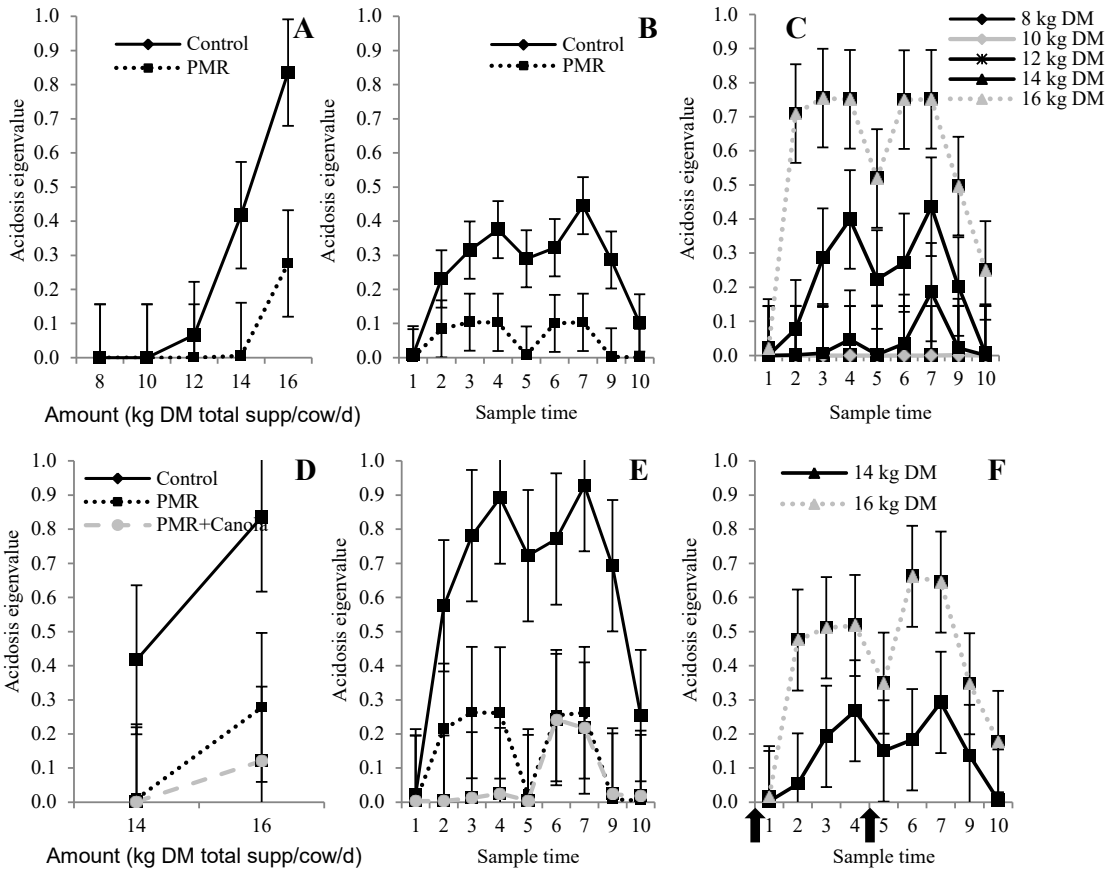

Figure 2. Mean (±SEM) acidosis eigenvalues for dairy cows from all feeding groups showing interactions between (A) feeding strategy and supplement feeding amount, (B) feeding strategy and sample time, and (C) supplement feeding amount and sample time. Mean (±SEM) acidosis eigenvalues for dairy cows from the high supplement feeding amount groups only (14 and 16z kg of DM of total supplement/cow per day) showing interactions between (D) feeding strategy and supplement feeding amount, (E) feeding strategy and sample time, and (F) supplement feeding amount and sample time. An eigenvalue of 0 corresponds to healthy, non-acidotic rumen sample and 1.0 represents an acidotic sample. Sample times were approximately 2.4 h apart over a 24-h period. Sample time 1 was approximately 8:20 h and milking was at 7:00 and 15:00 h (black arrows). PMR = partial mixed ration; PMR+Canola = partial mixed ration + canola meal; Amount = kg of DM of total supplement/cow per day. Sourced from Golder et al. (2014d).

Ruminal Ecology and Risk

The rumen is central to our understandings of cattle nutrition but is still largely unexplored, which is not too surprising given the large number of organisms present. Only a minority of the bacteria, archaea, viruses, fungi, and protozoa present in the rumen have been named or are able to be cultured, let alone their functions fully characterized. However, this field is rapidly changing with rapid sequencing of the DNA and rRNA or

rumen organisms, termed metataxomics, allowing investigations of the rumen environment to become more detailed (Jami et al., 2013). Recently, the effects of perturbing the rumen have been evaluated (Weimer et al., 2010; Golder et al., 2014b; Plaizier et al., 2017). Goldansaz et al. (2017) reviewed the opportunity for metabolomics, that is analytical techniques that can quantify small molecular weight products of metabolism, to be utilized in the investigation of production disease and examples of this include Loor et al. (2007) and Hailemariam et al. (2014). Metabolomics may be particularly powerful when used to evaluate responses to rumen perturbation (Ametaj et al., 2010b; Zhang et al., 2017). These new techniques are offering insights to the function and control of the rumen.

The *Bovidae,* including cattle, are among the most widely disseminated of the mammals. An important perspective can be obtained from a paper on the metataxome of the feces of mammals (Ley et al., 2008). This paper metatexamined similarities and differences in the fecal biota of a very diverse selection of mammals in the context of co-evolution of meta-taxomic communities. A key finding was that bacteria appear to be fairly promiscuous between hosts, a factor the authors speculated could account for the spectacular success of herbivores in general. The observations of Ley et al. (2008) are important to consider in the context of the way in which a species manages risk. In the case of cattle, times of abundance, for example lush legumes or abundant sugars or starches, or even toxic plants pose a risk to the animal and even a herd. This leads to a key understanding of the concept of a core rumen microbiome and a group of non-core organisms (Jami and Mizrahi, 2012; Lettat and Benchaar, 2013; Firkins and Yu, 2015). The core organisms appear to be common to most cattle in a group; however, there is very considerable diversity in the non-core (Zue et al., 2018). Perhaps the best example to consider is the protozoa that cattle maintain despite a high cost of predation of bacteria, leading to loss of approximately 20% in microbial protein outflow and lower average daily gain than defaunated cattle. However, these physiological responses are less for cattle on concentrate diets, suggesting an important role for protozoa in slowing the rate of starch degradation (Eugène et al., 2004) and a potentially valuable role in reducing the risk of acidosis. The adaptive responses of the rumen to severe dietary challenge; therefore, might be an expected variable, based on the concept that maintaining populations of organisms that may be less efficient but vital for survival, under particular challenge conditions, is a function of managing risk in a population.

Genome, meta-taxome, and function

Recent findings highlight the potential for further targeted manipulation of the rumen and the likelihood that acidosis is much more than a ruminal condition. These differences have been explained by different host genetics and interactions with the rumen meta-taxome (Weimer et al., 2010; King et al., 2011; Hernandez-Sanabria et al., 2013). Weimer et al. (2010) showed the ability of the rumen to revert to pre-exchange VFA concentration and rumen pH and nearly return to pre-exchange bacterial community composition within 24-hours of a 95% exchange of ruminal content with a cow on a similar diet. A second cow took a longer period to revert indicating the potential for variability in this response (Weimer et al., 2010).

Golder et al. (2018) demonstrated with limited numbers of cattle that there are strong links between the mammalian genome, the meta-taxome, and rumen function. There were several putative quantitative trait loci (**QTL**) identified for different metabolites. Five putative QTL were identified for the acetate to propionate ratio on chromosomes 1, 3, 5, 6, and 8. Eight putative QTL regions were identified for total lactate concentrations on chromosomes 1, 4, 6, 11, 22, and 24. Three putative QTL regions were identified for D-lactate concentrations on chromosomes 2, 8, and 26 and six putative QTL were identified for L-lactate concentrations on chromosomes 1, 4, 8, 17 and 24. One QTL was identified for the acidosis eigenvalue (this measure is obtained using the data from Bramley et al., 2008 and predicts how well the cows fits with an acidosis classification) on chromosome 19. Further, a large number of putative QTL were identified for bacterial phyla (Golder et al., 2018). Xue et al. (2018) evaluated the relationships between the meta-taxome and phenotypes for rumen function and production in over 300 cattle fed the same diet. They (Xue et al., 2018) found 6 phyla that represented over 45% of bacterial genotypes and included Firmicutes (21.67%), Bacteroidetes (20.68%), Proteobacteria (0.52%), Spirochaetes (1.35%), Fibrobacteres (0.86%), and Tenericutes (0.44%). There was marked animal variation in the prevalence of these taxa; however, relationships were identified among the bacteria, VFA, and production outcomes. These studies provide new insights that may allow better targeted nutrition and genetic selection in the future and provide a further basis to understand responses to perturbation of the rumen.

Perturbing the rumen

The primary methods used to perturb the rumen are feeding or administering single or multiple doses of RFCHO in the form of starches, sugars, or their combinations. Studies have noted considerable variation in responses among cattle fed a common diet designed to induce ruminal acidosis (Brown et al., 2000; Bevans et al., 2005; Penner et al., 2009; Golder et al., 2014b; Xue et al., 2018). Perturbation differences appear to be affected by genetic (Golder et al., 2018) and environmental factors (Xue et al., 2018) and likely their interactions (Golder et al., 2018). Substrate and other factors such as length of challenge and prior exposure to RFCHO etc affect rumen perturbation. Golder et al. (2012) fed non-pregnant Holstein heifers no grain or combinations of grain (1.2% of BW), fructose (0.4% of BW with 0.8% of BW grain), and histidine (6 g) in a single challenge feeding. It was evident that the rumen altered in response to the different substrates and substrate combinations. Heifers that had fructose included in their challenge ration had bacterial populations associated with increased total lactic acid and butyrate concentrations and decreased pH, while those that were not fed fructose had bacterial populations associated with the amount of grain consumed and ruminal ammonia, valerate, and histamine concentrations (Figure 3; Golder et al., 2014c).

In a longer-term challenge study, rumen perturbation increased with an increase in the amount of supplementary feeding and when isoenergetic diets included grain supplements fed in the milking parlor as opposed to supplements primarily fed in a mixed ration as shown by acidosis eigenvalues in Figure 2 for late lactation dairy cattle (Golder

et al., 2014d). Differences in associations between microbial populations and rumen metabolites between different groups of cattle fed differing amounts of supplement with these different feeding strategies are shown in Figure 4. Importantly, these findings show that substrate types (Figure 3) and amounts (Figure 4) determine the rumen populations and functions.

Further, Golder et al (2014b) fed pregnant heifers with a target DMI of 1.0% and 0.2% of BW of wheat and fructose, respectively with a non-fiber carbohydrate (**NFC**) content of 76.3% if 100% of the ration was consumed. These heifers had a 20-day exposure to total mixed rations including 10-days with an NFC content of 47.7% and a NFC of 46.1% prior to challenge. In contrast with the shorter challenge study with very similar amounts of grain and/or fructose (Golder et al., 2012) there were very large intra-group differences in rumen metabolites on the challenge day. Similarly, Firkins and Yu (2015) note that differences in the meta-taxome among animals within the same diet group often exceeds those among different diet groups.

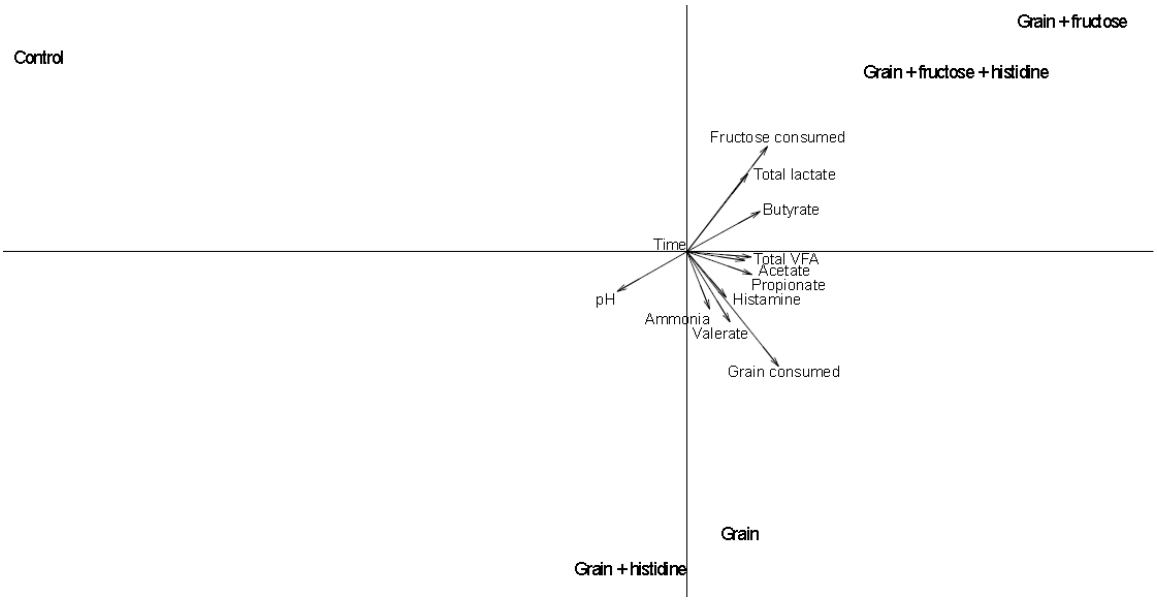

Figure 3. Duality diagram of co-inertia analysis of ruminal bacterial communities from 16S rDNA 454 pyrosequences, measures of ruminal fermentation, and percentages of offered grain and fructose from heifers that consumed the following single challenge rations: (1) control (no grain); (2) grain (1.2% of BW DM); (3) grain (1.2% of BW DM) + histidine (6 g/head); (4) grain (0.8% of BW DM) + fructose (0.4% of BW DM) or; (5) grain (0.8% of BW DM) + fructose (0.4% of BW DM) + histidine (6 g/head) (n of heifers = 6/group). Ruminal fluid was collected over approximately a 3.6-h period after (n of samples = 18/group). On the bi-plot the ruminal fermentation measures are represented as arrows. The direction of the arrow of each ruminal fermentation measure indicates an increasing concentration of that measure. The angle between the arrows indicates their degree of correlation. The magnitude of the arrows indicates the importance of the measure on the bacterial community composition. Measures with long arrows are more strongly correlated with the ordination axes than short arrows and have a greater influence on the pattern of variation (Carberry et al., 2012). Sourced from Golder et al. (2014c)

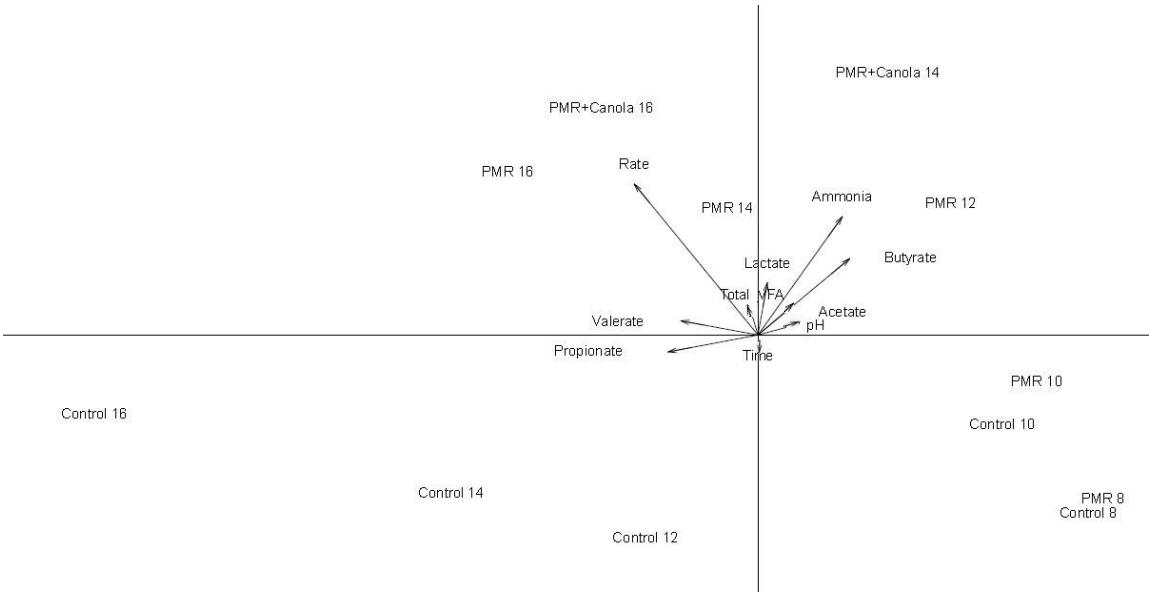

Figure 4. Duality diagram of co-inertia analysis of ruminal bacterial communities from 16S rDNA 454 pyrosequences, measures of ruminal fermentation, sample time, and amount of total supplements fed in dairy cattle fed 1 of 3 feeding strategies: control (n = 10 cows), partial mixed ration (PMR; n = 10 cows), or PMR+Canola (PMR+Canola meal n = 4 cows) at amounts 8, 10, 12, 14, or 16 kg of DM of total supplement/cow per day (2 cows per supplement feeding amount at 3 times from each feeding strategy). On the bi-plot the ruminal fermentation measures are represented as arrows. The direction of the arrow of each ruminal fermentation measure indicates an increasing magnitude of that measure. The angle between the arrows indicates their degree of correlation. The magnitude of the arrows indicates the importance of a measure on bacterial community composition. Measures with long arrows are more strongly correlated with the ordination axes than short arrows and have a greater influence on the pattern of variation (Carberry et al., 2012).

Controlling the Rumen

The studies outlined above, when combined with other literature provide the following clear guidelines to controlling the rumen.

Diet Form, Formulation, and Function

Consistency of supply of feed is important as many studies have withheld feed as part of a protocol to create acidosis (Nagaraja and Titgemeyer, 2007). Providing adequate fiber and particle length (Zebeli et al., 2012) and >30% NDF, based on Bramley et al. (2008) is appropriate for lactating dairy cattle. Diets formulated as partial mixed rations were safer, despite a higher NFC content, than diets that were component-fed (Golder et al., 2014c).

Sugars in the diet should be controlled based on Nagaraja et al. (1981) and (Golder et al., 2012b; Golder et al., 2014b). We suggest the following guidelines for TMR based on Bramley et al. (2008) and Golder et al. (2014d) for a maximum total NFC of 40 to 42%, 22 to 24% of starch, and 8% of sugar based on not exceeding approximately 0.35% of BW for sugars intake. It is very likely that not all sugars will have the same effect on the rumen (Plazier et al., 2018), and it is very evident that not all grains (Lean et al., 2013) or starches have the same effect on rumen function. Further, form of processing the concentrate components in the diet will influence function.

Lastly, observations that acidotic cattle have low rumen concentrations of ammonia (Bramley et al., 2008) and a reduction in the incidence and prevalence of acidosis with increased nitrogen in the diet (Golder et al., 2014d) support the observation that microbial protein is a significant sink for hydrogen in the rumen and that energy spilling ie an inability of bacteria to reproduce, hence produce more VFA, may be an important part of the pathogenesis of acidosis.

Feed additives

Buffers and Neutralizing Agents

These have been well reviewed and a buffer, by definition, reduces the decrease in pH without causing an increase in pH (Staples and Lough, 1989). Questions remain; however, in regard to the function of sodium bicarbonate, potassium carbonate, potassium bicarbonate, sodium sesquicarbonate, and the skeletal remains of the seaweed *Lithothamnium calcareum.* In the case of sodium bicarbonate, there are questions whether the effects are mediated through buffering the accumulated acid or increases in DM and water intakes caused by sodium, facilitated through an increased ruminal fluid dilution rate and reduced starch digestion rate (Russell and Chow, 1993; Valentine et al., 2000). Similarly, potassium-based products including potassium carbonate sesquihydrate, may be contributing to production increases through increased dietary cation anion difference or potassium requirements rather than through buffering actions. There are positive interactions for sodium bicarbonate with magnesium oxide and combination of sodium bicarbonate and magnesium oxide had similar effects as virginiamycin in controlling cyclic eating behaviour in cattle during adaptation to a diet high in grain and containing fructose (Golder et al., 2014b).

Antibiotics: While these are subject to regulatory change, there is strong evidence that some antibiotics can control the risk of acidosis (Lean et al., 2014). Tylosin has been widely used in finishing diets for the US beef industry. Virginiamycin is effective in controlling acidosis and tylosin, in combination with monensin, is also effective. It appears that combinations of monensin and bambermycin are also effective in favourably modifying rumen function. Both the latter are non-human class therapeutical agents.

Ionophores: Ionophores, particularly monensin and lasalocid are widely used in beef and dairy production. There is evidence of more sustained appetite (Lunn et al., 2005) and of increased production of propionate from lactate, which is a ruminal

adaptation that sequesters hydrogen ions in safer ruminal pools, when monensin is fed in diets that may cause acidosis. Monensin appears to be very effective in controlling acidosis risk when fed with tylosin or virginiamycin. Nagaraja et al. (1981) investigated the use of lasalocid to control lactic acidosis induced using finely ground corn or glucose. Use of lasalocid equalled or exceeded the reduction in lactic acid production observed for monensin (Nagaraja et al., 1981). Both monensin and lasalocid prevented acute lactic acidosis in the study of Nagaraja et al. (1981); however, both products were included in the diet at concentrations of 1.30 ppm of diet, and above concentrations recommended. Nagaraja et al. (1982) found that 0.33, 0.65, and 1.30 ppm of lasalocid were effective in reducing lactic acid concentrations and increasing pH compared to control cattle with lactic acidosis induced using glucose at 12.5 g/kg of BW. More studies would be useful to evaluate the effect of lasalocid on rumen acidosis.

Yeasts: There is increasing evidence that yeasts and yeast cultures may have a role in stabilizing rumen function. Actions that have been identified with live yeasts include small increases in rumen pH, reductions in lactic acid, enhanced fiber digestion, alterations in immune function and small increases in VFA production. These actions, are modest in magnitude, but may synergize with other strategies to control the risk of acidosis. Li et al. (2016) found that a *Saccharomyces cerevisiae* fermentation product stabilized rumen pH and Bach et al. (2018) demonstrated changes in immune markers in the epithelium and rumen to a live yeast. Weight gains and average daily gain improvements have been identified in beef receival cattle fed a hydrolyzed yeast (Salinas-Chavira et al., 2018) and reductions in severe liver abscess incidence also noted with an autolysed yeast (Ran et al., 2018). While these findings are encouraging, it is challenging to understand differences in the different yeast-based products and the best application of these in the field.

Probiotics: There is also some evidence that probiotics may provide benefits in terms of acidosis control; however, there are challenges in this area as candidate agents such as *Megasphera elsdenii* has not provided clear and consistent benefit in studies to date. It seems likely that more studies will investigate the roles of other agents in acidosis control in the future.

Conclusions

Acidosis is a much more complex condition than simply reflected in a drop in ruminal pH. Acidosis is increased by diets higher in starch and sugars and lower in fiber and is reflected in increases in propionate and valerate concentrations and reduced ammonia concentrations and rumen pH. While the clinical expression of acidosis may be influenced by the interactions of the gastrointestinal tract and immune system, we consider that prevention will depend on control of substrate and form and delivery of the diet. Better tests for acidosis will help identify, research, and manage the condition. These better tests, resulting in the more accurate identification of cattle with acidosis, will be critical to produce new interventions to assist in the control of acidosis in a higher percentage of the population. Recent developments in evaluating and understanding the

rumen and gastrointestinal tract function will provide new methods for controlling rumen function including selection of more production system adapted genotypes.

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Addressing Oxidative Stress in the Transition Cow and her Calf[1]

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Introduction

Dairy cattle can succumb to illnesses at any given time. However, the majority of diseases take place around to clusters: (1) the time around calving, commonly referred to as periparturient period, for metabolic and infectious diseases (e.g., ketosis, displaced abomasum, mastitis, metritis, etc.); and (2) the first few weeks of life, referred to as neonatal period, for diseases of calves (e.g., diarrhea or pneumonia). These periods of increased disease susceptibility are attributed to dysfunctional immune responses in these animals. Studies performed in the last decade clearly indicate that adult dairy cows experience oxidative stress (OS) around the time of calving (Castillo et al., 2003; 2005; 2006; Sordillo and Aitken, 2009; Abuelo et al., 2013, 2015). Also, some recent research has also documented that neonatal calves experience OS during the first few weeks of age (Gaal et al., 2006; Abuelo et al., 2014; Ranade et al., 2014). OS diminishes functional capabilities of immune cell populations and increases the animals' susceptibility to diseases (Sordillo and Aitken, 2009). In this presentation, the current knowledge regarding the impact of OS in these periods of increased disease incidence in dairy cattle populations.

Oxidative Stress vs. Oxidant Status

These terms have been used interchangeably in many instances. However, we now know that there is a clear difference between OS and oxidant status that should be considered. Oxidant status refers to the balance between the production of reactive oxygen/nitrogen species (ROS/RNS) and the total antioxidant capacity, whereas OS refers to the oxidative damage resulting from the imbalance between oxidants and antioxidants. OS includes oxidative modification of cellular macromolecules, cell death by apoptosis or necrosis, as well as structural tissue damage (Lykkesfeldt and Svendsen, 2007). It is expected that oxidative damage occurs as a result of shifts in the oxidant balance. Nevertheless, not all shifts in redox balance will result in OS, given that ROS/RNS are essential for many physiological processes and, therefore, changes in oxidant status might just reflect changes in redox signaling that are not associated with cell or tissue dysfunction.

This difference between OS and oxidant status also impacts the information that the different biomarkers provide. A review of the different methods available to measure OS or oxidant status is beyond the scope of this paper, and the readers are directed to

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previously published reviews (Palmieri and Sblendorio, 2007a, b; Celi, 2011a). However, it is important to understand whether the biomarker is an indicator of an oxidatively damaged molecule (e.g., isoprostanes, advanced oxidation protein products) or an indicator of the balance between pro- and antioxidants (e.g., Oxidant Status index (OSi); Abuelo et al. (2013))

Oxidative Stress in the Periparturient Period

Dairy cows go through dramatic physiological changes to prepare for the onset of lactation and peak milk production. In the peripartal cows, dry matter intake (DMI) decreases around parturition, whereas energy and calcium demands for lactation increase (Chapinal et al., 2012). In this situation, tissues consume more oxygen through normal cellular respiration during times of increased metabolic demand in order to provide the energy needed for the onset of lactation (Chapinal et al., 2012; Konvičná et al., 2015), resulting in energy deficit (ED). After calving, most cows undergo a period of ED, in which the energy demand for milk synthesis is not covered by feed intake. To meet the increased energy demands, cows mobilize body reserves predominantly from adipose tissue. Increased lipid mobilization as a consequence of ED may increase the generation ROS and RNS (Sordillo and Raphael, 2013; Celi and Gabai, 2015). An imbalance between both products coupled with the decreased intake of dietary antioxidants due to decreased overall feed intake can lead to a pro-oxidant shift in the redox balance that ultimately result in OS (Castillo et al., 2005; Dalle-Donne et al., 2005; Sordillo and Aitken, 2009). OS has been proposed as the nexus between the metabolic and immune systems of the cows during this stage (Sordillo and Mavangira, 2014; Abuelo et al., 2015).

Oxidative Stress and Periparturient Disease

Oxidative stress is a significant underlying factor to dysfunctional host immune and inflammatory responses that can increase the susceptibility of dairy cattle to a variety of health disorders, particularly during the transition period (Sordillo and Aitken, 2009). OS is known to diminish functional capabilities of immune cell populations and, therefore, increases the animals' susceptibility to infectious diseases during the periparturient period (Cemerski et al., 2003; Mehrotra et al., 2009; Abd Ellah et al., 2015). A more detailed description of the role of OS on periparturient cattle diseases is available elsewhere in these proceedings (Sordillo, 2019).

Owing to the role of OS in the pathophysiology of periparturient disease, biomarkers of OS and oxidant status have been proposed as potential predictors of disease (Celi, 2011b; Sordillo and Mavangira, 2014; Abuelo et al., 2015). Nevertheless, neither reference intervals nor cut-off points for OS biomarkers have yet been established to identify individual cows suffering from OS or to predict the likelihood of disease events or impairment of production outcomes at the herd level. Therefore, the application of these biomarkers in the field is still limited. Nevertheless, a recent study showed that biomarkers of oxidant status had a higher ability to predict fresh cow diseases at dry-off compared to commonly biomarkers of nutrient utilization such as nonesterified fatty acids, beta-hydroxybutyrate, calcium, etc. (Wisnieski et al., 2019). Thus, including biomarkers

of OS in herd monitoring protocols has the potential for allowing earlier detection of cows/cohorts at risk and to better inform nutritional management strategies such as antioxidant supplementation.

Preventing Periparturient Disease with Antioxidant Supplementation

In the literature, there are several strategies that have been proposed and tested as a method to avoid the development or at least minimize the development of OS status during the transition period (Lykkesfeldt and Svendsen, 2007; Politis, 2012; Abuelo et al., 2015). However, it should be noted that antioxidant supplementation has shown inconsistent results on dairy cows' health and production. Whilst most studies reported an improvement in health status or productivity, some studies have also shown no effect or even detrimental effects. The review of all the antioxidant supplementation studies is beyond the scope of this article and the readers should consult some of the relevant review articles (Politis, 2012; Abuelo et al., 2015). Here, only the underlying principle of most of these strategies is described: increasing the animals' antioxidant capacity so that it is better equipped to counteract the increase in free-radical production.

To inhibit impaired biological function due to damage to macromolecules by ROS/RNS, living organisms have developed a complex antioxidant defense system. Endogenous antioxidants can be divided into three major groups: enzymatic antioxidants, nonenzymatic protein antioxidants, and nonenzymatic low-molecular-weight antioxidants (Miller et al., 1993). Of these, the nonenzymatic antioxidants are primarily responsible for the antioxidant capacity of plasma. For example, the lipid-soluble α-tocopherol (vitamin E) protects cell membranes from lipid peroxidation; ascorbic acid (vitamin C) and β-carotene are able to quench singlet oxygen and peroxyl radicals and enhance the antioxidative effect of α-tocopherol. Other vitamins, such as retinol (vitamin A), only show antioxidant activity *in vitro*, but not *in vivo* (Azzi et al., 2004). Nevertheless, the study by LeBlanc et al. (2004) demonstrated that in the last week prepartum, a 100 ng/mL increase in serum retinol was associated with a 60% decrease in the risk of early lactation clinical mastitis. In addition, the authors observed significant positive associations of peripartum serum concentrations among α-tocopherol, β-carotene, and retinol.

In general terms, vitamins and certain trace minerals, such as selenium (Se), have been proven to be effective in counteracting OS and the severity of several dairy cattle diseases such as mastitis or metritis both through a direct antioxidant effect and by enhancing the immune response (Abuelo et al., 2015). Most of the established nutritional requirements traditionally focus on deficiency situations and there is now evidence that supplementation slightly above these reported requirements can improve animal health status and performance (Abuelo et al., 2015), as well as the quality of the final product (Castillo et al., 2013). Nevertheless, some studies reported deleterious effects of excessive antioxidant supplementation, such as the increase of odds for mastitis due to the increased production of ROS (Bouwstra et al., 2010a; 2010b). Hitherto, the level to which antioxidant supplementation stops being beneficial and starts to be associated with harmful consequences remains unknown. Hence, antioxidant supplementation strategies must be implemented only to levels slightly above current recommendations unless

strong scientific evidence is available to support its inclusion at a higher rate. The establishment of critical thresholds of OS biomarkers in periparturient dairy cattle will help inform more accurate antioxidant supplementation strategies.

Oxidative Stress in the Neonatal Period

The neonatal period of dairy calves is another time of increased disease susceptibility. Average neonatal morbidity and mortality rates are consistently reported above benchmarks worldwide, making high calf loss rates an international welfare problem (Mellor and Stafford, 2004; Mee, 2013). In the US dairy industry, pre-weaning morbidity and mortality rates are approximately 33 and 7-11%, respectively (NAHMS, 2014). As newborn calves adapt to the extra-uterine life, OS may contribute to increased disease susceptibility. However, redox biology also plays an important role in several physiological processes at this stage (Mutinati et al., 2014). As mentioned above for the periparturient period, it is the balance between the generation of ROS and the antioxidative capabilities of the animal that influence the development of OS and the subsequent development of systemic and localized dysfunctions. In the next sections we discuss different stages that lead to increased oxidant status during the neonatal period, as well as the knowledge available linking OS in calves with neonatal diseases and different prevention strategies.

In-Utero Conditions

The negative impact of metabolic stress on the immune function, health, and production of dairy cattle during this period is well established (Kehrli et al., 1989; Sordillo and Aitken, 2009). Metabolic stress starts several weeks before calving (Grummer, 1993; Sordillo and Raphael, 2013) and therefore can potentially affect the fetus. There is evidence in other non-ruminant species that maternal hypothalamic-pituitary-adrenal axis stress during gestation influences fetal development and exerts carryover effects on the offspring (McMillen and Robinson, 2005; Merlot et al., 2008). Studies in humans and murine models demonstrated that suboptimal intrauterine conditions during critical periods of development lead to changes in tissue structure and function (Fowden et al., 2006), that may have long-term consequences on the offspring's physiology and disease susceptibility (McMillen and Robinson, 2005; Merlot et al., 2008). Studies in ruminants have also demonstrated that exposure to heat stress and restricted or excessive energy intake during late gestation affects the immune and metabolic function of the offspring (Gao et al., 2012; Tao et al., 2012; Osorio et al., 2013; Tao et al., 2014; Yates et al., 2018). Moreover, Monteiro et al. (2016) demonstrated that the detrimental effects of in-utero exposure to heat stress on milk yield and reproductive performance extend to at least the first lactation of offspring. Thus, prenatal conditions have the potential of significantly impacting the productivity and health status of replacement heifers.

A recent study by Ling et al. (2018) compared the metabolic status and lipopolysaccharide (LPS)-induced whole blood TNFα release between calves born to cows that experienced different degrees of maternal metabolic stress during the last month of pregnancy. They found that calves born to cows with higher NEFA or OSi

showed lower bodyweight at birth and throughout the study, whilst no association between any of the maternal groups and average daily gain at 4 weeks of age was identified. Serum concentrations of ROS were higher in calves exposed to higher maternal NEFA concentrations or OSi when compared to calves born to cows with lower values of these biomarkers. Calves exposed to high maternal OS also had higher circulating concentrations of haptoglobin and TNFα, indicating greater basal inflammatory responses when compared to calves born to cows with a lower OSi. In contrast, LPS-induced inflammatory responses were less robust in calves exposed to higher maternal biomarkers of inflammation or OS, suggesting compromised immune responses to microbial agonists. Collectively, their results suggest that prenatal exposure to maternal parameters of metabolic stress (altered nutrient utilization, dysregulated inflammation, and OS) may adversely impact some metabolic and inflammatory responses of the offspring that could influence disease susceptibility. Hence, the metabolic stress experienced by periparturient cows not only predisposes the cows to transition cow disorders but also has carry-over effects on its offspring. However, further studies are still required to determine the clinical impact of these carry-over effects in the health and growth of the offspring to allow the development of adequate management practices. Nevertheless, some studies supplementing late-gestation cows with limiting amino acids or trace minerals have showed promising results in improving the immunometabolism of newborn calves (Jacometo et al., 2016), although the impact of such interventions in reducing calf morbidity and mortality rates remains unexplored.

The abovementioned study focused on the last month of pregnancy because this is when the time when maternal periparturient immune dysfunction starts and the period with the fastest proliferation of immune cells in the bovine fetus. Nevertheless, it still remains unexplored whether other critical windows of maternal metabolic stress exposure that can compromise the development of the fetal immune response exist. Similarly, it still needs to be elucidated in dairy cattle if OS is a key factor in adverse pregnancy outcomes as it has been reported in humans (Cuffe et al., 2017; Sultana et al., 2017).

The Oxidative Challenge of Birth

After birth, mammals are exposed for the first time to an oxygen rich environment once they start to breathe and this results in an increase in the production of ROS (Saugstad, 2003; Wiedemann et al., 2003). In humans, a brief oxygen exposure at birth induced a relatively long-lasting OS status (Saugstad, 2003). Hence, birth-associated OS might have relevant impacts in calves' cell growth, development, and death. Similar findings were identified in calves. Gaal et al. (2006) found that the concentration of ROS in calves' blood was 30% higher than in their dams shortly after birth and before colostrum ingestion. Given that pulmonary respiration and exposure to oxygen following birth are essential to maintain life, interventions to counteract birth-associated OS should focus on increasing the calves' pool of antioxidants.

Oxidant status during the pre-weaning period

A few studies have investigated the shifts in oxidant status during the first weeks of life in dairy calves. Gaal et al. (2006) noted that the blood concentration of free radicals was lower than day 1 at days 3 and 7 of age but increased again at 2 and 3 weeks of age. Conversely, other studies did not find and age effect in the concentration of ROS (Abuelo et al., 2014; Ranade et al., 2014). However, these studies used different biomarkers to assess pro-oxidant status. Nevertheless, Abuelo et al. (2014) indicated lower antioxidant status of newborn calves while they were being fed milk replacer but these changes in antioxidant potential were not found in the study by Ranade et al. (2014) where calves were fed whole milk until weaning. Milk replacers were found to have a low antioxidant capacity (Soberon et al., 2012). Thus, calves fed milk replacer might benefit from additional antioxidant supplementation.

Of particular interest is to note that biomarkers of oxidant status in calves were higher than those of periparturient cattle (Gaal et al., 2006; Abuelo et al., 2014). Hence, OS might play a very significant role in neonatal calf health. Indeed, OS is known to play a key role in the initiation and maintenance of important calf diseases such as diarrhea or pneumonia (Ranjan et al., 2006; Lykkesfeldt and Svendsen, 2007). A detailed description of the role of OS in disorders of ruminants is beyond the scope of this paper and readers are encouraged to consult the review by Celi (2011b) for this.

Another important factor to consider is the role of OS in the modulation of the immune response of newborn calves. There is currently solid evidence from *in vitro* and human studies proving that OS significantly impacts T lymphocyte functions (Figure 1): (1) polarization of T cell differentiation toward a Th2 phenotype (King et al., 2006), (2) T cell hyporesponsiveness to stimulation-induced activation (Cemerski et al., 2003), and (3) induction of apoptosis and inhibition of proliferation in T cells (Bhattacharyya et al., 2007; Thoren et al., 2007; Kasic et al., 2011).

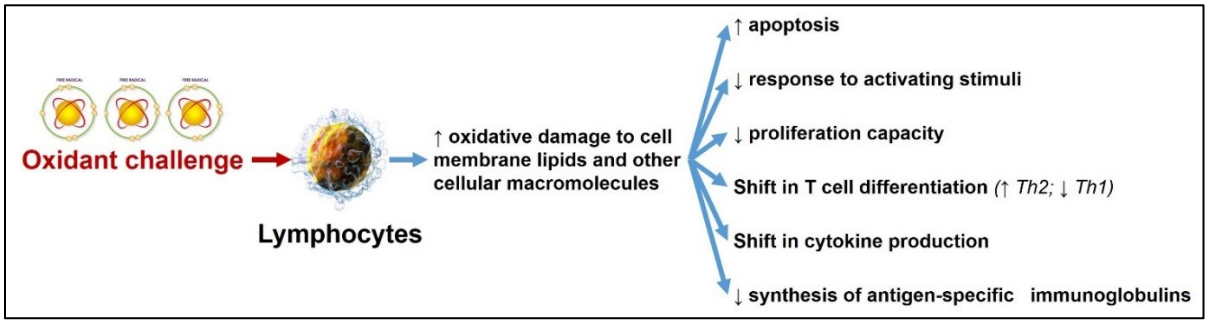

Figure 1: Schematic representation of the effect of prolonged exposure to ROS on human lymphocytes.

These functions of lymphocytes are essential for generating an adequate memory response and ensuring vaccination success. The degree of oxidant status experienced in newborn calves is also associated with differences in their profile of plasma circulating cytokines (Figure 2) and gene expression of key cytokines in PMBCs (Figure 3) (Abuelo and Sordillo, 2018).

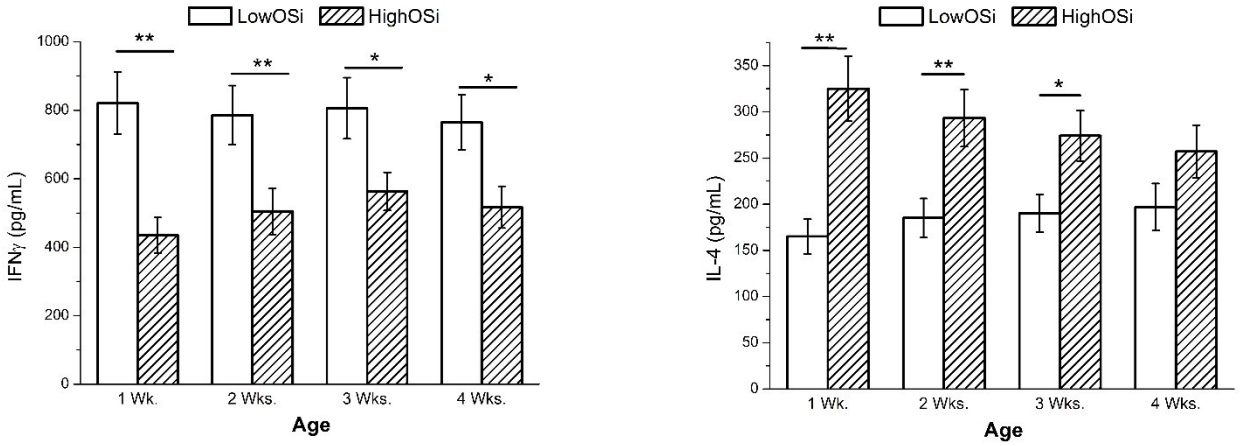

Figure 2. Plasma concentration of IFN-γ (left) and IL-4 (right) according to the calves' oxidant status throughout the first month of life. The oxidant status index (OSi) was monitored in sera of 12 healthy calves from the same farm by weekly blood sampling during their first month of life. Averages of OSi were calculated, and the calves were classified according to these values (Low OSi group = six lowest average OSi values). * $P < 0.05$, ** $P < 0.01$.

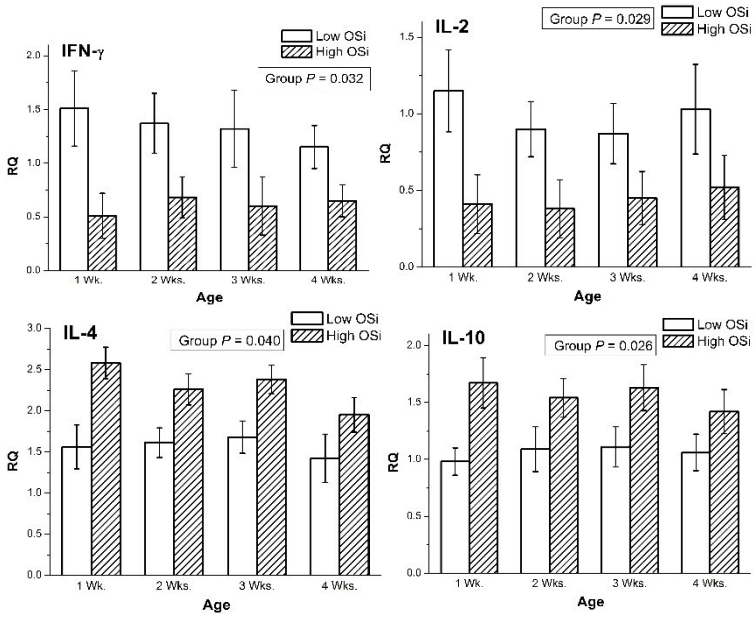

Figure 3. Relative gene expression of Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines in PBMCs of newborn calves according to the calves' oxidant status. (Low OSi = Lower degree of oxidant status; RQ = Relative Quantity)

These findings indicate that calves exposed to higher OS have an increased Th2 and reduced Th1 response in comparison to the calves exposed to a lower degree of OS. Although these data do not prove a causative role of OS in shifting the differentiation of T helper cells (↑ Th2, ↓ Th1), previous studies have demonstrated that OS promotes a polarization of human T cell differentiation toward the Th2 phenotype (King et al., 2006;

Kasic et al., 2011). Th2 responses are characterized by a reduced immune memory capacity that impacts the effectiveness of the immune response of newborn calves following immunization (Chase et al., 2008). Hence, it is critical to determine how OS alters key lymphocyte functions relevant for vaccine effectiveness and the extent to which these functions can be rescued with adequate antioxidant micronutrient supplementation.

Preventive measures and future research

As for periparturient cattle, several strategies exist to decrease the risk of OS in neonatal calves. Below, some of the most common ones are summarized, identifying some of the gaps in knowledge that are still present:

Maternal Supplementation with Antioxidants

Supplementation of antioxidants during the dry period slightly above NRC (2001) requirements has shown beneficial effects for cow health and productivity (Abuelo et al., 2015). However, given the carry-over effect of maternal OS on neonatal metabolic and immune function, this practice can also have beneficial effects in the offspring. However, research proving the effects of maternal antioxidant supplementation on calf morbidity and mortality rates is, to the best of the author's knowledge, non-existing to date.

In humans, antenatal supplementation of antioxidant vitamins and minerals has long been a recommended practice to reduce OS at delivery (Bolisetty et al., 2002; Scaife et al., 2006). Some studies in cattle have also shown that dry-period antioxidant supplementation enhances the antioxidative profile of newborn calves (Abdelrahman and Kincaid, 1995; Horn et al., 2010; Jacometo et al., 2015). Nevertheless, various factor limit this route in cattle: (1) the epitheliochorial nature of the ruminants' placenta limits the types of antioxidants that can be transmitted transplacentally, (2) dry dairy cattle are usually already supplemented with considerable amounts of some antioxidants (e.g., selenium close to the US legal limit of 0.3 ppm) for the prevention of transition diseases, (3) excessive antioxidant supplementation can have downstream effects in the health of dairy cattle (Bouwstra et al., 2010a; Bouwstra et al., 2010b; Abuelo et al., 2015) and has been linked with stillbirths in humans (Joshi et al., 2008). Hence, dry cows should not receive antioxidants in amounts significantly exceeding the NRC (2001) requirements.

Colostrum: A Source of Antioxidants and Pro-Oxidants

The importance of colostrum ingestion to the health of the neonatal calf has been well-known for several decades (Besser and Gay, 1994). However, this has been primarily attributed to the acquisition of passive immunity (immunoglobulins) to infectious diseases with calves experiencing failure of passive transfer showing decreased survival rates on farms compared to those with adequate blood immunoglobulin concentrations (Godden, 2008). In addition to immunoglobulins, colostrum is also rich in other beneficial substances such as immune cells, growth factors, cytokines, etc. (Stelwagen et al., 2009). Given that colostrum is the first meal that a calf should receive shortly after birth, its antioxidant content is important to offset the birth-associated OS. However, compared to normal milk, colostrum has the same amount of oxidants but less antioxidants, with the

concentration of the latter increasing progressively from the first milked colostrum onwards (Kankofer and Lipko-Przybylska, 2008; Albera and Kankofer, 2011). Hence, colostrum provides antioxidants to calves but is also a source of pro-oxidants. Nevertheless, newborn calves seem to be able to counter effectively the birth-associated OS (Gaal et al., 2006), with calves showing a gradual decrease in oxidant status biomarkers (Inanami et al., 1999; Albera and Kankofer, 2011). Indeed, Abuelo et al. (2014) found that 2h after colostrum ingestion, calves showed the lowest OSi values of the first months of life. To the best of our knowledge, however, no study has hitherto compared the redox balance between calves that ingest colostrum shortly after birth with those experiencing delayed colostrum ingestion. Hence, it remains unexplored whether this gradual decline in OS following birth is due to the transfer of antioxidants via colostrum, the activation of antioxidative pathways in the calves, or a combination of both.

In addition, colostrum redox balance seems to play a role in immunoglobulin absorption. Selenium supplementation of colostrum increases immunoglobulin absorption (Kamada et al., 2007), and the colostrum redox profile was significantly associated with calves' serum immunoglobulin concentrations (Abuelo et al., 2014). However, none of these studies demonstrated which mechanisms might be implicated and therefore further research is needed. Also, a negative association between colostrum immunoglobulin content and antioxidant capacity has been reported (Abuelo et al., 2014). The authors attributed this finding to a consumption of antioxidants in protecting from peroxidation the highly susceptible immunoglobulins during the colostrogenesis process. Therefore, supplementation of colostrum with antioxidants seems to have additional benefits to the calf beyond counteracting the birth-associated OS. Also, there is now a plethora of research indicating the long-term impact of early-life events and management in the calves' productive life once they reach maturity (Kertz et al., 2017). Hence, studies investigating the long-term implications of supplementation of colostrum with antioxidants in the animals' health and productivity are also required.

Supplementation of Calves with Antioxidants

Other ways of increasing the antioxidant potential of calves are the parenteral or dietary administration of vitamins and trace elements. This is a routine management practice in many farms within the first days of life. It's been well-stablished that vitamin supplementation of dairy calves can increase their performance, metabolism, and immune system (Reddy et al., 1985; Reddy et al., 1986; Reddy et al., 1987a; Reddy et al., 1987b). Parenteral trace mineral supplementation (zinc, selenium, manganese, and copper) at 3 and 30 days of life resulted in increased neutrophil function and glutathione peroxidase activity and decreased incidence of health disorders when compared to the control group (Teixeira et al., 2014). Also, trace mineral supplementation concurrent with a polyvalent viral vaccine administration at 30 days of age resulted in improved cell-mediated immune responses (Palomares et al., 2016). However, whether this observed increase improves the vaccine's protection against infection remains unknown.

It is important to note, however, that the NRC (2001) requirements were initially developed to prevent deficiencies and there are no clear guidelines of the levels of

antioxidant supplementation for optimized performance. Considering that it is likely that as happens in adult cows, excessive antioxidant supplementation can have detrimental effects on calf health and performance, caution must be exerted when supplementing antioxidants above levels deemed safe by the scientific literature. Indeed, there are reports of toxicosis due to excessive supplementation (MacDonald et al., 1981). This might be even more relevant for those antioxidants, such as selenium, that can be transferred via the placenta when the dams are also supplemented.

Summary

Redox balance is essential for several biological processes of dairy cows and calves. However, when an imbalance exists between the production of pro-oxidants and the animals' antioxidant abilities, OS can develop, and this has been associated with immune and metabolic dysfunction. Also, in pregnant animals, the degree of OS experienced not only puts the dams at risk of subsequent diseases during the onset of lactation, but also have an impact on the offspring. However, antioxidant therapy is capable of protecting against OS-conditions, and several methods for delivery of antioxidants are routinely used in dairy farms. Nevertheless, the findings have been inconsistent at times with some studies not showing an effect. Hence, more research is still needed to provide evidence-based guidance on levels and timing of supplementation that provide an effective improvement of the animals' health status.

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Addressing Oxidative Stress in the Transition Cow and her Calf[1]

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Introduction

Dairy cattle can succumb to illnesses at any given time. However, the majority of diseases take place around to clusters: (1) the time around calving, commonly referred to as periparturient period, for metabolic and infectious diseases (e.g., ketosis, displaced abomasum, mastitis, metritis, etc.); and (2) the first few weeks of life, referred to as neonatal period, for diseases of calves (e.g., diarrhea or pneumonia). These periods of increased disease susceptibility are attributed to dysfunctional immune responses in these animals. Studies performed in the last decade clearly indicate that adult dairy cows experience oxidative stress (OS) around the time of calving (Castillo et al., 2003; 2005; 2006; Sordillo and Aitken, 2009; Abuelo et al., 2013, 2015). Also, some recent research has also documented that neonatal calves experience OS during the first few weeks of age (Gaal et al., 2006; Abuelo et al., 2014; Ranade et al., 2014). OS diminishes functional capabilities of immune cell populations and increases the animals' susceptibility to diseases (Sordillo and Aitken, 2009). In this presentation, the current knowledge regarding the impact of OS in these periods of increased disease incidence in dairy cattle populations.

Oxidative Stress vs. Oxidant Status

These terms have been used interchangeably in many instances. However, we now know that there is a clear difference between OS and oxidant status that should be considered. Oxidant status refers to the balance between the production of reactive oxygen/nitrogen species (ROS/RNS) and the total antioxidant capacity, whereas OS refers to the oxidative damage resulting from the imbalance between oxidants and antioxidants. OS includes oxidative modification of cellular macromolecules, cell death by apoptosis or necrosis, as well as structural tissue damage (Lykkesfeldt and Svendsen, 2007). It is expected that oxidative damage occurs as a result of shifts in the oxidant balance. Nevertheless, not all shifts in redox balance will result in OS, given that ROS/RNS are essential for many physiological processes and, therefore, changes in oxidant status might just reflect changes in redox signaling that are not associated with cell or tissue dysfunction.

This difference between OS and oxidant status also impacts the information that the different biomarkers provide. A review of the different methods available to measure OS or oxidant status is beyond the scope of this paper, and the readers are directed to

[1] Portions of this work were published at: Abuelo *et al.* 2019. Redox biology in transition periods of dairy cattle: Role in the health of periparturient and neonatal animals. *Antioxidants (Basel)* 8(1):20. doi:10.3390/antiox8010020

previously published reviews (Palmieri and Sblendorio, 2007a, b; Celi, 2011a). However, it is important to understand whether the biomarker is an indicator of an oxidatively damaged molecule (e.g., isoprostanes, advanced oxidation protein products) or an indicator of the balance between pro- and antioxidants (e.g., Oxidant Status index (OSi); Abuelo et al. (2013))

Oxidative Stress in the Periparturient Period

Dairy cows go through dramatic physiological changes to prepare for the onset of lactation and peak milk production. In the peripartal cows, dry matter intake (DMI) decreases around parturition, whereas energy and calcium demands for lactation increase (Chapinal et al., 2012). In this situation, tissues consume more oxygen through normal cellular respiration during times of increased metabolic demand in order to provide the energy needed for the onset of lactation (Chapinal et al., 2012; Konvičná et al., 2015), resulting in energy deficit (ED). After calving, most cows undergo a period of ED, in which the energy demand for milk synthesis is not covered by feed intake. To meet the increased energy demands, cows mobilize body reserves predominantly from adipose tissue. Increased lipid mobilization as a consequence of ED may increase the generation ROS and RNS (Sordillo and Raphael, 2013; Celi and Gabai, 2015). An imbalance between both products coupled with the decreased intake of dietary antioxidants due to decreased overall feed intake can lead to a pro-oxidant shift in the redox balance that ultimately result in OS (Castillo et al., 2005; Dalle-Donne et al., 2005; Sordillo and Aitken, 2009). OS has been proposed as the nexus between the metabolic and immune systems of the cows during this stage (Sordillo and Mavangira, 2014; Abuelo et al., 2015).

Oxidative Stress and Periparturient Disease

Oxidative stress is a significant underlying factor to dysfunctional host immune and inflammatory responses that can increase the susceptibility of dairy cattle to a variety of health disorders, particularly during the transition period (Sordillo and Aitken, 2009). OS is known to diminish functional capabilities of immune cell populations and, therefore, increases the animals' susceptibility to infectious diseases during the periparturient period (Cemerski et al., 2003; Mehrotra et al., 2009; Abd Ellah et al., 2015). A more detailed description of the role of OS on periparturient cattle diseases is available elsewhere in these proceedings (Sordillo, 2019).

Owing to the role of OS in the pathophysiology of periparturient disease, biomarkers of OS and oxidant status have been proposed as potential predictors of disease (Celi, 2011b; Sordillo and Mavangira, 2014; Abuelo et al., 2015). Nevertheless, neither reference intervals nor cut-off points for OS biomarkers have yet been established to identify individual cows suffering from OS or to predict the likelihood of disease events or impairment of production outcomes at the herd level. Therefore, the application of these biomarkers in the field is still limited. Nevertheless, a recent study showed that biomarkers of oxidant status had a higher ability to predict fresh cow diseases at dry-off compared to commonly biomarkers of nutrient utilization such as nonesterified fatty acids, beta-hydroxybutyrate, calcium, etc. (Wisnieski et al., 2019). Thus, including biomarkers

of OS in herd monitoring protocols has the potential for allowing earlier detection of cows/cohorts at risk and to better inform nutritional management strategies such as antioxidant supplementation.

Preventing Periparturient Disease with Antioxidant Supplementation

In the literature, there are several strategies that have been proposed and tested as a method to avoid the development or at least minimize the development of OS status during the transition period (Lykkesfeldt and Svendsen, 2007; Politis, 2012; Abuelo et al., 2015). However, it should be noted that antioxidant supplementation has shown inconsistent results on dairy cows' health and production. Whilst most studies reported an improvement in health status or productivity, some studies have also shown no effect or even detrimental effects. The review of all the antioxidant supplementation studies is beyond the scope of this article and the readers should consult some of the relevant review articles (Politis, 2012; Abuelo et al., 2015). Here, only the underlying principle of most of these strategies is described: increasing the animals' antioxidant capacity so that it is better equipped to counteract the increase in free-radical production.

To inhibit impaired biological function due to damage to macromolecules by ROS/RNS, living organisms have developed a complex antioxidant defense system. Endogenous antioxidants can be divided into three major groups: enzymatic antioxidants, nonenzymatic protein antioxidants, and nonenzymatic low-molecular-weight antioxidants (Miller et al., 1993). Of these, the nonenzymatic antioxidants are primarily responsible for the antioxidant capacity of plasma. For example, the lipid-soluble α-tocopherol (vitamin E) protects cell membranes from lipid peroxidation; ascorbic acid (vitamin C) and β-carotene are able to quench singlet oxygen and peroxyl radicals and enhance the antioxidative effect of α-tocopherol. Other vitamins, such as retinol (vitamin A), only show antioxidant activity *in vitro*, but not *in vivo* (Azzi et al., 2004). Nevertheless, the study by LeBlanc et al. (2004) demonstrated that in the last week prepartum, a 100 ng/mL increase in serum retinol was associated with a 60% decrease in the risk of early lactation clinical mastitis. In addition, the authors observed significant positive associations of peripartum serum concentrations among α-tocopherol, β-carotene, and retinol.

In general terms, vitamins and certain trace minerals, such as selenium (Se), have been proven to be effective in counteracting OS and the severity of several dairy cattle diseases such as mastitis or metritis both through a direct antioxidant effect and by enhancing the immune response (Abuelo et al., 2015). Most of the established nutritional requirements traditionally focus on deficiency situations and there is now evidence that supplementation slightly above these reported requirements can improve animal health status and performance (Abuelo et al., 2015), as well as the quality of the final product (Castillo et al., 2013). Nevertheless, some studies reported deleterious effects of excessive antioxidant supplementation, such as the increase of odds for mastitis due to the increased production of ROS (Bouwstra et al., 2010a; 2010b). Hitherto, the level to which antioxidant supplementation stops being beneficial and starts to be associated with harmful consequences remains unknown. Hence, antioxidant supplementation strategies must be implemented only to levels slightly above current recommendations unless

strong scientific evidence is available to support its inclusion at a higher rate. The establishment of critical thresholds of OS biomarkers in periparturient dairy cattle will help inform more accurate antioxidant supplementation strategies.

Oxidative Stress in the Neonatal Period

The neonatal period of dairy calves is another time of increased disease susceptibility. Average neonatal morbidity and mortality rates are consistently reported above benchmarks worldwide, making high calf loss rates an international welfare problem (Mellor and Stafford, 2004; Mee, 2013). In the US dairy industry, pre-weaning morbidity and mortality rates are approximately 33 and 7-11%, respectively (NAHMS, 2014). As newborn calves adapt to the extra-uterine life, OS may contribute to increased disease susceptibility. However, redox biology also plays an important role in several physiological processes at this stage (Mutinati et al., 2014). As mentioned above for the periparturient period, it is the balance between the generation of ROS and the antioxidative capabilities of the animal that influence the development of OS and the subsequent development of systemic and localized dysfunctions. In the next sections we discuss different stages that lead to increased oxidant status during the neonatal period, as well as the knowledge available linking OS in calves with neonatal diseases and different prevention strategies.

In-Utero Conditions

The negative impact of metabolic stress on the immune function, health, and production of dairy cattle during this period is well established (Kehrli et al., 1989; Sordillo and Aitken, 2009). Metabolic stress starts several weeks before calving (Grummer, 1993; Sordillo and Raphael, 2013) and therefore can potentially affect the fetus. There is evidence in other non-ruminant species that maternal hypothalamic-pituitary-adrenal axis stress during gestation influences fetal development and exerts carryover effects on the offspring (McMillen and Robinson, 2005; Merlot et al., 2008). Studies in humans and murine models demonstrated that suboptimal intrauterine conditions during critical periods of development lead to changes in tissue structure and function (Fowden et al., 2006), that may have long-term consequences on the offspring's physiology and disease susceptibility (McMillen and Robinson, 2005; Merlot et al., 2008). Studies in ruminants have also demonstrated that exposure to heat stress and restricted or excessive energy intake during late gestation affects the immune and metabolic function of the offspring (Gao et al., 2012; Tao et al., 2012; Osorio et al., 2013; Tao et al., 2014; Yates et al., 2018). Moreover, Monteiro et al. (2016) demonstrated that the detrimental effects of in-utero exposure to heat stress on milk yield and reproductive performance extend to at least the first lactation of offspring. Thus, prenatal conditions have the potential of significantly impacting the productivity and health status of replacement heifers.

A recent study by Ling et al. (2018) compared the metabolic status and lipopolysaccharide (LPS)-induced whole blood TNFα release between calves born to cows that experienced different degrees of maternal metabolic stress during the last month of pregnancy. They found that calves born to cows with higher NEFA or OSi

showed lower bodyweight at birth and throughout the study, whilst no association between any of the maternal groups and average daily gain at 4 weeks of age was identified. Serum concentrations of ROS were higher in calves exposed to higher maternal NEFA concentrations or OSi when compared to calves born to cows with lower values of these biomarkers. Calves exposed to high maternal OS also had higher circulating concentrations of haptoglobin and TNFα, indicating greater basal inflammatory responses when compared to calves born to cows with a lower OSi. In contrast, LPS-induced inflammatory responses were less robust in calves exposed to higher maternal biomarkers of inflammation or OS, suggesting compromised immune responses to microbial agonists. Collectively, their results suggest that prenatal exposure to maternal parameters of metabolic stress (altered nutrient utilization, dysregulated inflammation, and OS) may adversely impact some metabolic and inflammatory responses of the offspring that could influence disease susceptibility. Hence, the metabolic stress experienced by periparturient cows not only predisposes the cows to transition cow disorders but also has carry-over effects on its offspring. However, further studies are still required to determine the clinical impact of these carry-over effects in the health and growth of the offspring to allow the development of adequate management practices. Nevertheless, some studies supplementing late-gestation cows with limiting amino acids or trace minerals have showed promising results in improving the immunometabolism of newborn calves (Jacometo et al., 2016), although the impact of such interventions in reducing calf morbidity and mortality rates remains unexplored.

The abovementioned study focused on the last month of pregnancy because this is when the time when maternal periparturient immune dysfunction starts and the period with the fastest proliferation of immune cells in the bovine fetus. Nevertheless, it still remains unexplored whether other critical windows of maternal metabolic stress exposure that can compromise the development of the fetal immune response exist. Similarly, it still needs to be elucidated in dairy cattle if OS is a key factor in adverse pregnancy outcomes as it has been reported in humans (Cuffe et al., 2017; Sultana et al., 2017).

The Oxidative Challenge of Birth

After birth, mammals are exposed for the first time to an oxygen rich environment once they start to breathe and this results in an increase in the production of ROS (Saugstad, 2003; Wiedemann et al., 2003). In humans, a brief oxygen exposure at birth induced a relatively long-lasting OS status (Saugstad, 2003). Hence, birth-associated OS might have relevant impacts in calves' cell growth, development, and death. Similar findings were identified in calves. Gaal et al. (2006) found that the concentration of ROS in calves' blood was 30% higher than in their dams shortly after birth and before colostrum ingestion. Given that pulmonary respiration and exposure to oxygen following birth are essential to maintain life, interventions to counteract birth-associated OS should focus on increasing the calves' pool of antioxidants.

Oxidant status during the pre-weaning period

A few studies have investigated the shifts in oxidant status during the first weeks of life in dairy calves. Gaal et al. (2006) noted that the blood concentration of free radicals was lower than day 1 at days 3 and 7 of age but increased again at 2 and 3 weeks of age. Conversely, other studies did not find and age effect in the concentration of ROS (Abuelo et al., 2014; Ranade et al., 2014). However, these studies used different biomarkers to assess pro-oxidant status. Nevertheless, Abuelo et al. (2014) indicated lower antioxidant status of newborn calves while they were being fed milk replacer but these changes in antioxidant potential were not found in the study by Ranade et al. (2014) where calves were fed whole milk until weaning. Milk replacers were found to have a low antioxidant capacity (Soberon et al., 2012). Thus, calves fed milk replacer might benefit from additional antioxidant supplementation.

Of particular interest is to note that biomarkers of oxidant status in calves were higher than those of periparturient cattle (Gaal et al., 2006; Abuelo et al., 2014). Hence, OS might play a very significant role in neonatal calf health. Indeed, OS is known to play a key role in the initiation and maintenance of important calf diseases such as diarrhea or pneumonia (Ranjan et al., 2006; Lykkesfeldt and Svendsen, 2007). A detailed description of the role of OS in disorders of ruminants is beyond the scope of this paper and readers are encouraged to consult the review by Celi (2011b) for this.

Another important factor to consider is the role of OS in the modulation of the immune response of newborn calves. There is currently solid evidence from *in vitro* and human studies proving that OS significantly impacts T lymphocyte functions (Figure 1): (1) polarization of T cell differentiation toward a Th2 phenotype (King et al., 2006), (2) T cell hyporesponsiveness to stimulation-induced activation (Cemerski et al., 2003), and (3) induction of apoptosis and inhibition of proliferation in T cells (Bhattacharyya et al., 2007; Thoren et al., 2007; Kasic et al., 2011).

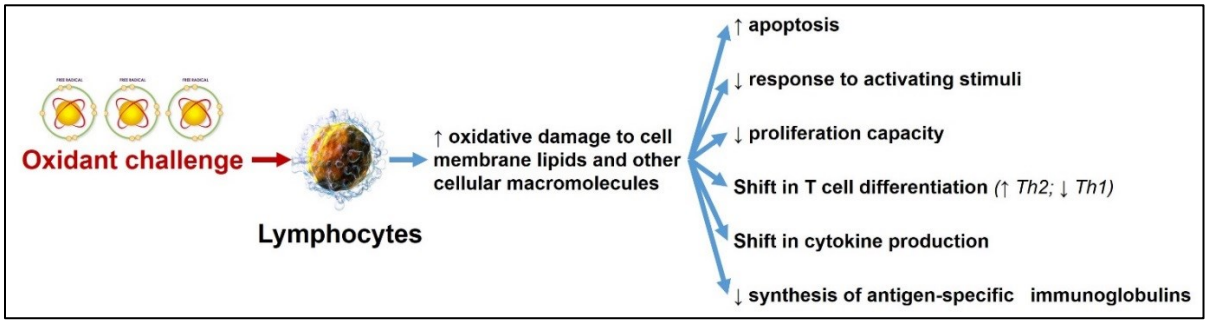

Figure 1: Schematic representation of the effect of prolonged exposure to ROS on human lymphocytes.

These functions of lymphocytes are essential for generating an adequate memory response and ensuring vaccination success. The degree of oxidant status experienced in newborn calves is also associated with differences in their profile of plasma circulating cytokines (Figure 2) and gene expression of key cytokines in PMBCs (Figure 3) (Abuelo and Sordillo, 2018).

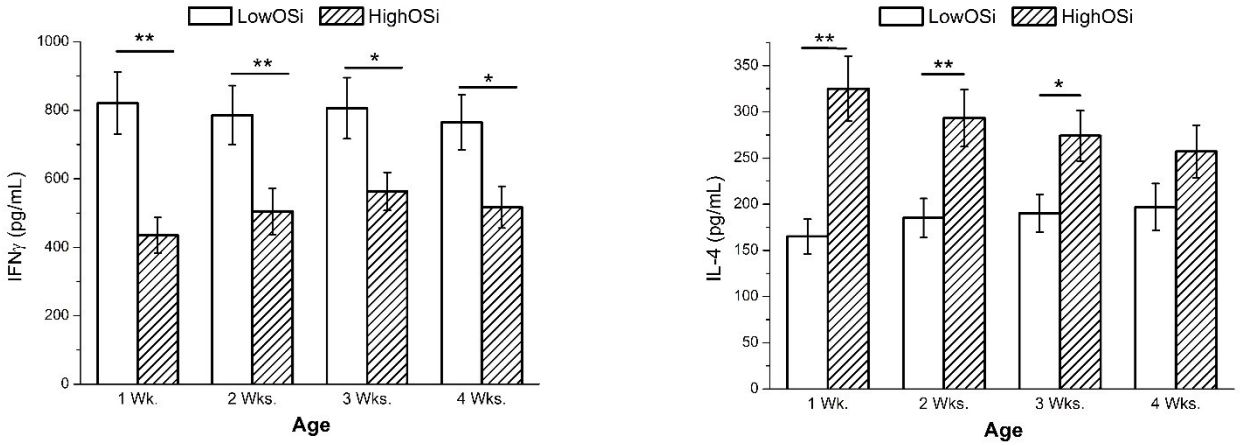

Figure 2. Plasma concentration of IFN-γ (left) and IL-4 (right) according to the calves' oxidant status throughout the first month of life. The oxidant status index (OSi) was monitored in sera of 12 healthy calves from the same farm by weekly blood sampling during their first month of life. Averages of OSi were calculated, and the calves were classified according to these values (Low OSi group = six lowest average OSi values). * $P < 0.05$, ** $P < 0.01$.

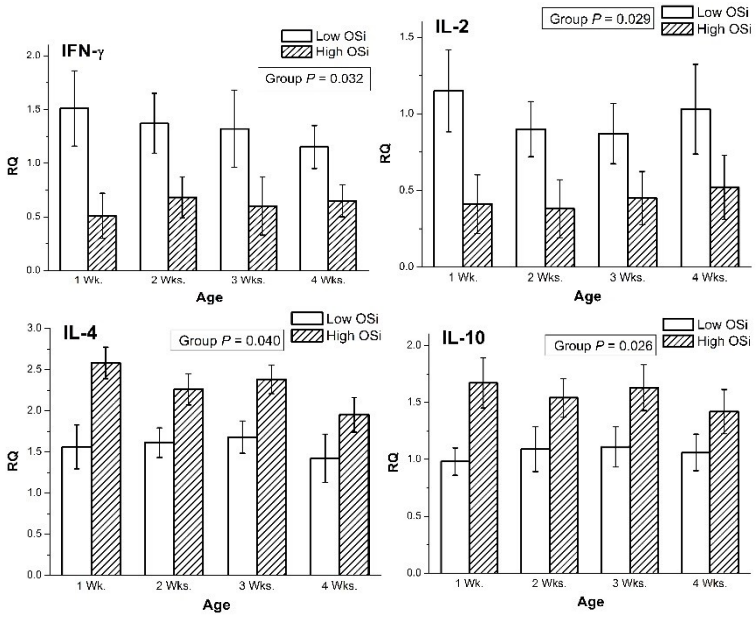

Figure 3. Relative gene expression of Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines in PBMCs of newborn calves according to the calves' oxidant status. (Low OSi = Lower degree of oxidant status; RQ = Relative Quantity)

These findings indicate that calves exposed to higher OS have an increased Th2 and reduced Th1 response in comparison to the calves exposed to a lower degree of OS. Although these data do not prove a causative role of OS in shifting the differentiation of T helper cells (↑ Th2, ↓ Th1), previous studies have demonstrated that OS promotes a polarization of human T cell differentiation toward the Th2 phenotype (King et al., 2006;

Kasic et al., 2011). Th2 responses are characterized by a reduced immune memory capacity that impacts the effectiveness of the immune response of newborn calves following immunization (Chase et al., 2008). Hence, it is critical to determine how OS alters key lymphocyte functions relevant for vaccine effectiveness and the extent to which these functions can be rescued with adequate antioxidant micronutrient supplementation.

Preventive measures and future research

As for periparturient cattle, several strategies exist to decrease the risk of OS in neonatal calves. Below, some of the most common ones are summarized, identifying some of the gaps in knowledge that are still present:

Maternal Supplementation with Antioxidants

Supplementation of antioxidants during the dry period slightly above NRC (2001) requirements has shown beneficial effects for cow health and productivity (Abuelo et al., 2015). However, given the carry-over effect of maternal OS on neonatal metabolic and immune function, this practice can also have beneficial effects in the offspring. However, research proving the effects of maternal antioxidant supplementation on calf morbidity and mortality rates is, to the best of the author's knowledge, non-existing to date.

In humans, antenatal supplementation of antioxidant vitamins and minerals has long been a recommended practice to reduce OS at delivery (Bolisetty et al., 2002; Scaife et al., 2006). Some studies in cattle have also shown that dry-period antioxidant supplementation enhances the antioxidative profile of newborn calves (Abdelrahman and Kincaid, 1995; Horn et al., 2010; Jacometo et al., 2015). Nevertheless, various factor limit this route in cattle: (1) the epitheliochorial nature of the ruminants' placenta limits the types of antioxidants that can be transmitted transplacentally, (2) dry dairy cattle are usually already supplemented with considerable amounts of some antioxidants (e.g., selenium close to the US legal limit of 0.3 ppm) for the prevention of transition diseases, (3) excessive antioxidant supplementation can have downstream effects in the health of dairy cattle (Bouwstra et al., 2010a; Bouwstra et al., 2010b; Abuelo et al., 2015) and has been linked with stillbirths in humans (Joshi et al., 2008). Hence, dry cows should not receive antioxidants in amounts significantly exceeding the NRC (2001) requirements.

Colostrum: A Source of Antioxidants and Pro-Oxidants

The importance of colostrum ingestion to the health of the neonatal calf has been well-known for several decades (Besser and Gay, 1994). However, this has been primarily attributed to the acquisition of passive immunity (immunoglobulins) to infectious diseases with calves experiencing failure of passive transfer showing decreased survival rates on farms compared to those with adequate blood immunoglobulin concentrations (Godden, 2008). In addition to immunoglobulins, colostrum is also rich in other beneficial substances such as immune cells, growth factors, cytokines, etc. (Stelwagen et al., 2009). Given that colostrum is the first meal that a calf should receive shortly after birth, its antioxidant content is important to offset the birth-associated OS. However, compared to normal milk, colostrum has the same amount of oxidants but less antioxidants, with the

concentration of the latter increasing progressively from the first milked colostrum onwards (Kankofer and Lipko-Przybylska, 2008; Albera and Kankofer, 2011). Hence, colostrum provides antioxidants to calves but is also a source of pro-oxidants. Nevertheless, newborn calves seem to be able to counter effectively the birth-associated OS (Gaal et al., 2006), with calves showing a gradual decrease in oxidant status biomarkers (Inanami et al., 1999; Albera and Kankofer, 2011). Indeed, Abuelo et al. (2014) found that 2h after colostrum ingestion, calves showed the lowest OSi values of the first months of life. To the best of our knowledge, however, no study has hitherto compared the redox balance between calves that ingest colostrum shortly after birth with those experiencing delayed colostrum ingestion. Hence, it remains unexplored whether this gradual decline in OS following birth is due to the transfer of antioxidants via colostrum, the activation of antioxidative pathways in the calves, or a combination of both.

In addition, colostrum redox balance seems to play a role in immunoglobulin absorption. Selenium supplementation of colostrum increases immunoglobulin absorption (Kamada et al., 2007), and the colostrum redox profile was significantly associated with calves' serum immunoglobulin concentrations (Abuelo et al., 2014). However, none of these studies demonstrated which mechanisms might be implicated and therefore further research is needed. Also, a negative association between colostrum immunoglobulin content and antioxidant capacity has been reported (Abuelo et al., 2014). The authors attributed this finding to a consumption of antioxidants in protecting from peroxidation the highly susceptible immunoglobulins during the colostrogenesis process. Therefore, supplementation of colostrum with antioxidants seems to have additional benefits to the calf beyond counteracting the birth-associated OS. Also, there is now a plethora of research indicating the long-term impact of early-life events and management in the calves' productive life once they reach maturity (Kertz et al., 2017). Hence, studies investigating the long-term implications of supplementation of colostrum with antioxidants in the animals' health and productivity are also required.

Supplementation of Calves with Antioxidants

Other ways of increasing the antioxidant potential of calves are the parenteral or dietary administration of vitamins and trace elements. This is a routine management practice in many farms within the first days of life. It's been well-stablished that vitamin supplementation of dairy calves can increase their performance, metabolism, and immune system (Reddy et al., 1985; Reddy et al., 1986; Reddy et al., 1987a; Reddy et al., 1987b). Parenteral trace mineral supplementation (zinc, selenium, manganese, and copper) at 3 and 30 days of life resulted in increased neutrophil function and glutathione peroxidase activity and decreased incidence of health disorders when compared to the control group (Teixeira et al., 2014). Also, trace mineral supplementation concurrent with a polyvalent viral vaccine administration at 30 days of age resulted in improved cell-mediated immune responses (Palomares et al., 2016). However, whether this observed increase improves the vaccine's protection against infection remains unknown.

It is important to note, however, that the NRC (2001) requirements were initially developed to prevent deficiencies and there are no clear guidelines of the levels of

antioxidant supplementation for optimized performance. Considering that it is likely that as happens in adult cows, excessive antioxidant supplementation can have detrimental effects on calf health and performance, caution must be exerted when supplementing antioxidants above levels deemed safe by the scientific literature. Indeed, there are reports of toxicosis due to excessive supplementation (MacDonald et al., 1981). This might be even more relevant for those antioxidants, such as selenium, that can be transferred via the placenta when the dams are also supplemented.

Summary

Redox balance is essential for several biological processes of dairy cows and calves. However, when an imbalance exists between the production of pro-oxidants and the animals' antioxidant abilities, OS can develop, and this has been associated with immune and metabolic dysfunction. Also, in pregnant animals, the degree of OS experienced not only puts the dams at risk of subsequent diseases during the onset of lactation, but also have an impact on the offspring. However, antioxidant therapy is capable of protecting against OS-conditions, and several methods for delivery of antioxidants are routinely used in dairy farms. Nevertheless, the findings have been inconsistent at times with some studies not showing an effect. Hence, more research is still needed to provide evidence-based guidance on levels and timing of supplementation that provide an effective improvement of the animals' health status.

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Dietary Lecithin Supplementation in Dairy Cattle

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Introduction

Lecithins are sourced from animals and plants for the food, feed additive, pharmaceutical and cosmetic industries. Lecithins function as emulsifiers, fillers, viscosity regulators, carriers, wetting, anti-spattering and dispersing agents. Their amphiphilic nature (polar/hydrophilic headgroup and nonpolar/lipophilic tail) affords them properties that allow them to accumulate at the interface of oil and water, thus reducing interfacial tension and enhancing the formation of emulsions. Lecithin feeding is common practice for non-ruminants and the pre-ruminant calf; however, lecithin-based feed additives have received less attention for growing and lactating ruminants because of their susceptibility to rumen modification. This conference proceeding aims to review fundamental concepts in lipid digestion and absorption in the ruminant. The production, composition, and emulsifying properties of lecithin are summarized. The effects of phospholipids on rumen function, and the ability of lecithin or lysolecithin feeding to modulate milk production and composition is discussed. A recent comprehensive study at Cornell University that investigated the effects of dietary deoiled soy lecithin supplementation on milk production, fatty acid digestibility, and choline availability in Holstein cows fed fractionated palm fatty acids is also presented.

Definitions

Lecithin: Phospholipids (or glycerophospholipids) of animal or plant origin that contain two hydrophobic hydrocarbon tails and a hydrophilic head group. Lecithin most often exists as a mixture of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol. Phosphatidylserine, phosphatidylglycerol, and phosphatidic acid may also be present in a lesser amount.

Lysolecithin: Lysophospholipids of animal or plant origin that contain a single hydrocarbon tail and a polar head group. Lysolecithin is generally recognized as being lysophosphatidylcholine (LPC).

Crude lecithin: A mixture of triglycerides (~35%) and phospholipids (~45%) that also includes lesser amounts of glycolipids, sterols, and carbohydrates. Although variable by source, PC content is often 15 to 20%.

Deoiled lecithin: A mixture of phospholipids with triglycerides removed. Phospholipid composition varies based on origin; however, PC content is generally 20 to 30%.

Fractionated lecithin: A purified form enriched in one or more types of a specific phospholipid (e.g., >50% PC).

Modified lecithin: Lecithin with a modified structure (e.g., caused by hydrogenation or partial hydrolysis).

Lipid Digestion and Absorption in Ruminants

Esterified lipids, like dietary triglycerides, undergo extensive hydrolysis in the rumen; albeit, the magnitude of hydrolysis is influenced by rumen pH, ionophores, and level of dietary fat as reviewed by Bauman and Lock (2006). Extensive rumen hydrolysis explains why the majority of lipid that enters the small intestine exist in the free fatty acid form. Rumen lipid metabolism also involves hydrogenation of unsaturated fatty acids. Extensive biohydrogenation of 18-carbon oleic, linoleic, and linolenic acids explains why the flow of stearic acid to the duodenum is high even though stearic acid intake is low (Harvatine and Allen, 2006). Short-chain fatty acid absorption occurs in the rumen epithelium, where they serve as key energy substrate molecules for oxidative metabolism in the mature ruminant. In contrast, the absorption of long-chain fatty acids primarily occurs in the small intestine, which is due in part to their adsorption on feed particles prior to entry. The composition of long-chain fatty acids in duodenal digesta mostly reflects the rumen profile and is responsive to changes in dietary saturated and unsaturated fatty acid feeding (Harvatine and Allen, 2006).

A key feature of lipid digestion is micelle formation. Mixed micelles contain bile acids and salts, lecithin, lysolecithin, monoglycerides, cholesterol, and fatty acids that ensure lipid absorption across the unstirred water layer at the surface of the intestinal microvillus membrane. Micelle formation occurs after complex lipid digestion and emulsification. Pancreatic juice and bile aide in this capacity. Pancreatic juice is composed of digestive enzymes and bicarbonate. The digestive enzymes include proteases (i.e., trypsin and chymotrypsin), lipases, and amylase. With regard to lipid digestion, pancreatic lipase and colipase are proteins synthesized and secreted by the pancreas. Lipase catalyzes the hydrolysis of triglycerides. Colipase aides in triglyceride digestion because it is a required co-factor for pancreatic lipase. Interestingly, colipase is a cleavage product of a precursor molecule called procolipase via the actions of trypsin in the intestine. Pancreatic juice also contains pancreatic phospholipases A_1 and A_2. Phospholipase A_1 hydrolyzes the mainly saturated fatty acids located at position 1 of the phospholipid to form 2-acyl-lysolecithin. Phospholipase A_1 hydrolyzes the mainly unsaturated fatty acid in position 2. A similar product of triglyceride and phospholipid digestion is the fatty acid. Monoglyceride and lysolecithin are also formed, which have emulsifying properties and are absorbed by the intestine (the latter being more relevant in the ruminant).

It is important to recognize that the ability of pancreatic lipase, colipase, and phospholipases to efficiently digest dietary triglycerides and phospholipids (i.e., gain access) in the ruminant requires the emulsifying and micelle-forming properties of bile salts and lysolecithin. Bile production begins in the liver. Bile is subsequently modified by

absorptive and secretory transport systems in the bile duct epithelium and concentrated in the gallbladder before delivery to the intestinal lumen. The composition of bile is complex but includes water with dissolved bile salts, lecithin, lysolecithin, and sphingomyelin (i.e., phospholipids; Figure 1A). Cholesterol, amino acids, and vitamins are also present. Pancreatic phospholipase may convert bile-derived PC to LPC. Bile salts are conjugated bile acids and products of cholesterol metabolism. Examples include cholic, taurocholic, glycocholic, chenodeoxycholic, and glycodeoxycholic acids (Karsai and Szaniszló, 1990; Washizu et al., 1991). In dairy cattle, major lecithin phospholipids of bile include PC (e.g., PC 18:0/18:2; Figure 1A), LPC (e.g., LPC 18:2), and sphingomyelin (e.g., SM 34:1). In a recycling manner, bile salts may be de-conjugated by bacteria in the small intestine to reform bile acids that may be reabsorbed in the terminal ileum to complete enterohepatic circulation.

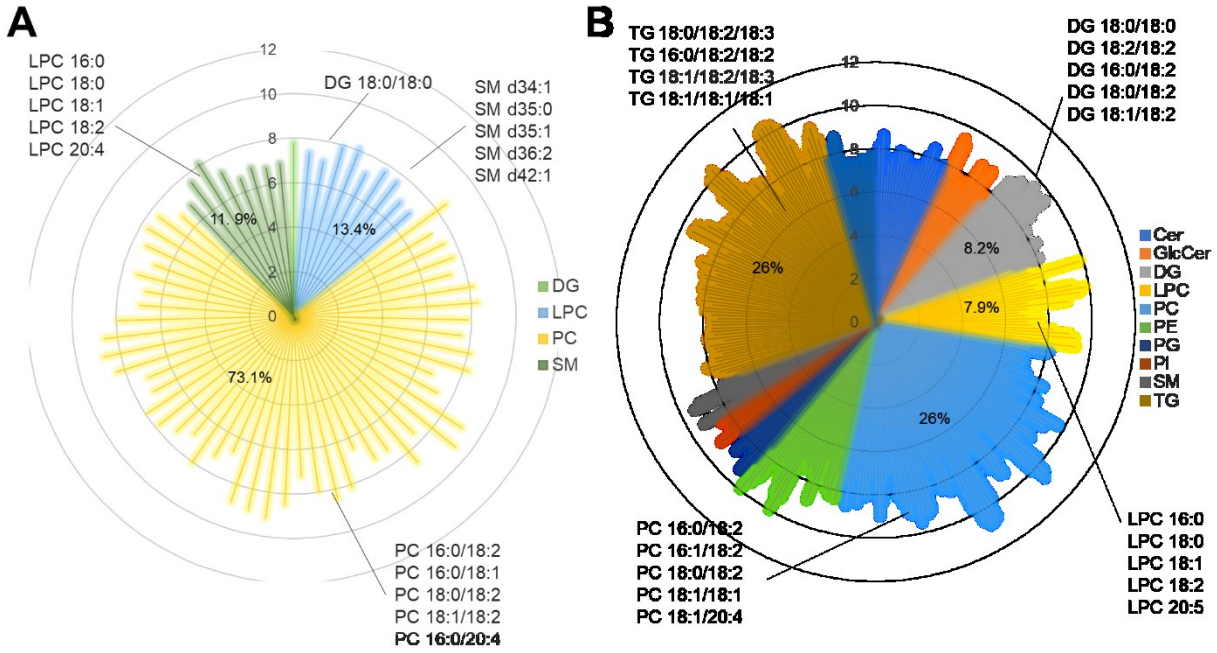

Figure 1. Lipid profiles for bovine bile (A) and deoiled soy lecithin (B). Annotated data were generated in positive mode using time-of-flight mass spectrometry. Each line represents a lipid detected with relative abundance. Examples are shown. Cer = ceramide, DG = diglyceride; GlcCer = monohexosylceramide; PG = phosphatidylglycerol; PI = phosphatidylinositol; SM = sphingomyelin; TG = triglyceride.

Unsaturated fatty acids that enter the small intestine or released from triglyceride or phospholipid digestion may also aide in micelle formation. Specifically, Freeman (1969) investigated the effects of different amphiphiles on triglyceride dispersion and fatty acid solubility in a bile salt (i.e., sodium glycodeoxycholate) solution. It was confirmed that palmitic and stearic acids behave as non-polar solutes, whereas, oleic, linoleic, and lauric acids were amphiphilic. Increasing the concentration of each unsaturated fatty acid amphiphile enhanced stearic acid solubility; however, stearic acid solubility was greatest in the presence of lysolecithin, relative to oleic, linoleic, and lauric acids as well as monoglyceride. These data would suggest that lecithin hydrolysis in the small intestine

would release lysolecithin and unsaturated fatty acids (e.g., oleic or linoleic acids) and thus have an additive effect on saturated fatty acid digestibility. This uncertainty requires consideration in the cow.

At the brush border membrane of the small intestine (jejunum primarily), lipid absorption into the enterocyte occurs by simple diffusion or transporter proteins. Long-chain fatty acids and monoglycerides are utilized to re-synthesize triglyceride. Long-chain fatty acids and LPC serve as substrate for PC synthesis. Triglyceride, cholesterol, and PC are packaged with apoproteins (e.g., apo-B48) into particles called chylomicrons. Chylomicrons are then secreted from the basolateral membrane of the enterocyte into lacteals of the lymphatic system. Chylomicron-containing lymph then empties into venous blood circulation at the thoracic duct. This route of delivery ensures that triglycerides are utilized by the mammary gland, skeletal muscle, and heart prior to hepatic entry. Short- and medium-chain fatty acids are absorbed into the portal vein as free fatty acids.

Production, Composition and Emulsifying Properties of Lecithin

Derived from the Greek term ‘lekithos’ (i.e., egg yolk; Hensing, 2004), lecithin is a generic term to define amphiphilic phospholipids from animals or plants. The types of glycerophospholipids in lecithin include PC, PE, phosphatidylinositol, phosphatidylserine, and their lyso-phospholipid counterparts such as LPC. Lecithin distributed for commercial application is predominantly derived from soybeans but also sunflower seed and rapeseed. In addition to plant-based lecithins, animal-derived egg or milk lecithin is also available for human food applications, which includes emulsification, wetting, dispersing and texturization. To obtain lecithin from plants, processing is required. Degumming is the method of removing phosphatides from extracted crude vegetable oil with water to form a gum that contains phospholipids, triglycerides, free fatty acids, and glycolipids. Removal of water generates a liquid crude lecithin that is primarily composed of phospholipid (~45%) but still enriched in triglycerides (~35-40%). Washing the crude lecithin with acetone removes the neutral oil (i.e., the triglycerides, fatty acids, and sterols) to produce deoiled lecithin composed of phospholipids (~70-80%) and a small percentage of glycolipids (i.e., acetone insoluble compounds). Deoiled lecithin is dried by evaporation to produce a phospholipid-enriched lecithin powder. The glycerophospholipid composition of deoiled lecithin depends on the source; however, PC, PE, and phosphatidylinositols are key examples. The presence of lyso-phospholipids and triglycerides is expected to be negligible in deoiled lecithin (<5%). The fatty acid composition of crude or deoiled lecithin certainly depends on the vegetable source. A recent analysis of deoiled soy lecithin (BergaPur®; Berg+Schmidt GmbH & Co, Hamburg, Germany) by gas chromatography reported 54% C18:2, 22% C16:0, and 13% C18:1 of total fatty acids (unpublished data). A lipidomic evaluation of deoiled soy lecithin revealed an abundance of PC, LPC, and sphingomyelin (Figure 1B). Many of these lipids were enriched in linoleic and palmitic acids. Lecithin derived from sunflower seeds or rapeseed would be expected to be enriched in linoleic and oleic acids, respectively. Although more common in non-agricultural industries, deoiled lecithin may be fractionated by organic solvent extraction to separate PC from other glycerophospholipids. Moreover, purified lecithin can be chemically modified by hydroxylation, halogenation, acetylation,

hydrolysis, hydrogenation, phosphorylation, or sulfonation. For example, partial hydrolysis transforms lecithin (e.g., PC) into lysolecithin (e.g., LPC). For additional information, detailed reviews of soybean and sunflower lecithin have been prepared by List (2015) and Guiotto et al. (2015).

The chemical structure of phospholipids influence their emulsifying properties. In a comparison of phospholipids, emulsion droplet size is lowest for LPC and PC, relative to phosphatidic acid, sphingomyelin, or PE (Ishii, 1992). Other work confirmed that lysolecithin forms smaller emulsion oil droplets, relative to lecithin in mock enteral preps (Shimokawa et al., 2017), and the size of mixed micelles containing lysolecithin are smaller than that of lecithin micelles when studied in comparable molar ratios (Reynier et al., 1985). Replacement of lecithin with lysolecithin has been shown to increase the solubilization of palmitic acid in an aqueous solution containing bile salts (Lough and Smith, 1976). Lysolecithin is more water soluble than lecithin. Regardless, saturation of lecithin has the potential to influence emulsification. Saturated PC with C12:0 or C14:0 produce glyceryl trioctanoate emulsion droplets of smaller size, relative to PC containing C16:0 or C18:0 (Nii and Ishii, 2004; Ishii and Nii, 2005). Interestingly, a small droplet size prepared with PC enriched in C12:0 or C14:0 is comparable to C18:1- or C18:2-linked PC (Nii and Ishii, 2004). In experiments studying egg yolk lecithin, mean droplet diameter for triglyceride emulsions tended to increase with degree of saturation of PC with 18-carbon chains (i.e., PC containing C18:2 > 18:1 > 18:0). We should consider the interaction between lecithin and the type of neutral triglyceride emulsified. As triglyceride carbon number (i.e., chain length) increases (e.g., tricaprylin, tricaprin, trilaurin, and trimyristin), mean diameter droplet size also increases (Nii and Ishii, 2005). This has been shown to occur more so in emulsion preps containing dipalmitoyl- or distearoyl-PC, relative to dilauroyl- or dimyristoyl-PC (Nii and Ishii, 2005). As lecithin or lysolecithin-based feed additives become commercially available for the livestock industry, the composition of phospholipid deserves consideration.

Lecithin and Lysolecithin Feeding in Ruminants

Early studies demonstrated that crude lecithin and lysolecithin are degraded to glycerylphosphorylcholine and fatty acids in ovine rumen fluid by phospholipase and lysophospholipase (Dawson, 1959; Hazlewood and Dawson, 1975; Jenkins, 1993). Subsequent work confirmed that deoiled soy lecithin is degraded in ovine rumen cultures (Jenkins et al., 1989). In sheep, supplementing crude soybean lecithin reduced energy, acid detergent fiber and nitrogen digestibility (Jenkins and Fotouhi, 1990). In vitro, lecithin from canola (deoiled/hydrolysed/acetylated) or soy (deoiled) has been shown to lower total volatile fatty acid and ammonia concentrations, and apparent ruminal degradation of organic matter and crude protein, relative to unsupplemented controls (Wettstein et al., 2000b). In beef calves, dietary supplementation of increasing amounts of a mixture containing soybean hulls, soy lecithin, and soapstock (0 to 7% supplemental fat on a DM basis; in replace of soybean hulls only) resulted in a linear decrease in the in situ rate of ruminal neutral detergent fiber (NDF) digestion with no effect on the rate of crude protein digestion (Shain et al., 1993). In lactating cows, lower apparent digestibility of dry matter, organic matter and gross energy were observed with deoiled soy lecithin feeding, relative

to animals fed calcium soaps of palm fatty acids (Wettstein et al., 2000a). This body of work would suggest that unprotected lecithin feeding modifies rumen fermentation and nutrient digestibility in ruminants.

The study of dietary lecithin supplementation on fatty acid digestibility in poultry and livestock is warranted because amphiphiles are required for the efficient absorption of saturated fat (Freeman, 1969). In broilers, dietary LPC supplementation improved total tract digestibility of palmitic, oleic, and linoleic acids (Zhang et al., 2011). In weanling pigs, lecithin (or soy oil) feeding increased fatty acid digestibility, relative to a no added fat control (Øverland et al., 1993). In similar manner, the addition of lecithin improved ether extract digestibility in weaned pigs fed tallow (Jin et al., 1998). However, lysophospholipid feeding did not modify apparent ileal digestibility of fatty acids in ileal-cannulated growing pigs (Dierick and Decuypere, 2004). The authors postulated that the reason may be related to a high ratio of unsaturated to saturated fatty acids of the animal fat source fed in the base diet. Most studies in ruminants have not observed improvements in fatty acid digestibility with unprotected lecithin feeding. In Angus steers fed hydrogenated fats that are highly saturated, dietary lecithin supplementation did not modify fatty acid digestibility (Jenkins, 1990). Replacement of dietary rumen-protected fat (calcium soaps of palm oil fatty acids) with lecithin (raw, deoiled and deoiled/partially hydrolysed soy lecithin, and raw canola lecithin) did modify total fatty acid digestibility in Brown Swiss dairy cows (Wettstein et al., 2000a). More recently, supplementing lactating Holstein cows with oleic acid with lecithin increased total and 16-carbon fatty acid digestibility, relative to cows fed a control diet containing prills of saturated fat; however, the response was not shown with lecithin alone (Shepardson and Harvatine, 2019). An exception is a study of Hampshire wethers fed soy lecithin that tended to increase fatty acid digestion in the hindgut, relative to no added fat (Jenkins and Fotouhi, 1990). It can be hypothesized that rumen phospholipid degradation may prevent their use as emulsifiers in the lower gut; however, interpreting the described studies is complicated considering that the triglyceride and phospholipid composition for phospholipid-based feed additives is often not described. Research needs to focus on the effects of the processed form (e.g., crude vs deoiled) and composition (e.g., PC content) of phospholipid feed additives on fatty acid digestibility. Of equal importance, studies need to evaluate the effects of post-ruminal lecithin delivery of intestinal 16- and 18-carbon fatty acid digestibility and absorption. The development of technologies that protect lecithin and lysolecithin from ruminal degradation are a necessity.

Feeding lecithin and lysolecithin modifies lactation performance in ruminants. In Holstein dairy cattle fed isonitrogenous and isoenergetic diets, efficiency of 4% fat-corrected milk production was greatest for cows fed the same soybean hull, soy lecithin, and soapstock mixture at an intermediate feeding level (Shain et al., 1993). The abomasal infusion of soy lecithin (33% soy oil, 20% PC, 20% PE, and 21% phosphatidylinositol) increased 3.5% fat-corrected milk, and milk fat content and yield in lactating cows, relative to water or soy oil infusion; although dry matter intake was reduced by lecithin infusion, relative to water infusion (no change in milk yield). The authors postulated that abomasal lecithin infusion may have increased lipid digestion and intestinal fatty acid uptake for their utilization by the mammary gland. Increased supply of post-ruminant linoleic acid

from soy lecithin digestion could increase the incorporation of this fatty acid in milk. In support, feeding lactating cows a ration containing soy lecithin and soapstock resulted in higher milk C18:2 content, relative to cows fed a diet containing soybean oil (Abel-Caines et al., 1998). The effects of lysophospholipids on milk production have also been considered in dairy cattle. In lactating cows, lysolecithin supplementation increased milk fat concentration when lactating cows were fed a higher fiber and lower unsaturated fatty acid diet, but lowered milk fat yield when a lower fiber and higher unsaturated fatty acid diet was fed (Rico et al., 2017). In the same study, lysolecithin feeding lowered dry matter intake when cows were fed the lower fiber and higher oil diet; however, lysolecithin treatment did not appear to modify rumen biohydrogenation of unsaturated fatty acids. This may be due to a limited feeding level of lysolecithin (10 g/cow per day). In a different study, supplementation of lysophospholipids linearly increased milk yield, milk fat and protein yields, and feed efficiency to produce milk, relative to an unsupplemented control diet; however, total tract digestibility of dry matter and organic matter tended to be lower (Lee et al., 2019). In this investigation cows were fed a hydrolyzed soy lecithin-based product that included a nominal amount of lysophospholipids (6%), and the base diet contained ~33% NDF (% of ration dry matter) and hydrolyzed tallow instead of soybean oil. These findings by Rico et al. (2017) and Lee et al. (2019) suggests that feeding unprotected phospholipids is best for cows fed diets adequate in NDF with limited unsaturated fat. However, the effects of lecithin or lysolecithin supplementation at varying feeding levels, and the interactions between phospholipid feeding and the fatty acid content and composition of the base diet deserves further evaluation in dairy cattle.

Dietary Lecithin Supplementation in Dairy Cows Re-Visited

The McFadden lab recently completed a comprehensive study of dietary lecithin supplementation on milk production and composition, markers of metabolic health, plasma and milk fatty acid concentrations, and apparent fatty acid digestibility in Holstein cows fed fractionated palm fatty acids (Fontoura et al., 2019; Rico et al., 2019). In a split-plot Latin square design, sixteen Holstein cows (160 ± 7 DIM) were randomly allocated to a main plot receiving a corn silage and alfalfa haylage-based diet with palm fat containing either moderate or high palmitic acid content at 1.75% of ration DM (MPA and HPA, respectively; BergaFat® F-100 Classic or F-100 HP containing 87 or 98% PA, respectively; Berg + Schmidt, Hamburg, Germany; n = 8 per group). On each palm fat diet, deoiled soy lecithin was top-dressed at 0, 0.12, 0.24, or 0.36% of ration DM in a replicated 4 × 4 Latin Square design (0 to ~100 g/cow per d; BergaPur®). Following a 14 d covariate period, lecithin treatment spanned 14 d with milk and blood collected during the final 3 d of each experimental period. Milk composition was determined. Pooled serum and plasma were used to measure markers of metabolic health such as total fatty acids and liver enzymes. Milk, feed, fecal and plasma fatty acid concentrations were determined by gas chromatography. Nutrient digestibility was calculated using indigestible NDF as an internal marker. Choline-related metabolites were quantified using liquid chromatography and mass spectrometry. Untargeted lipidomics was employed for plasma lipid profiling using quadrupole time-of-flight mass spectrometry. The statistical model included the fixed effects of palm fat type, lecithin level, period, and their interactions as well as the random effect of cow.

Lecithin linearly decreased dry matter intake (29.2, 28.7, 27.0 and 27.3 kg/d, P = 0.01). In cows fed HPA, lecithin feeding decreased milk fat content (interaction, P < 0.01) and tended to lower milk fat yield (interaction, P = 0.10). Although no changes in milk yield were observed, a quadratic reduction in 3.5% fat-corrected milk was observed with increasing lecithin supplementation (P = 0.001). Interestingly, lecithin linearly increased efficiency to produce energy-corrected milk in cows fed MPA (P < 0.05). The proportion of 16C fatty acids in milk fat decreased linearly with lecithin level, whereas 18C fatty acids increased linearly (e.g., 18:0; P < 0.01). The milk fat content of de novo fatty acids was lowered by lecithin (P < 0.05). Lecithin feeding decreased circulating milk urea nitrogen concentrations, relative to unsupplemented cows (0 vs rest, P = 0.01) and linearly increased total serum fatty acid concentrations (P = 0.01). Lecithin supplementation did not overtly modify the concentrations of individual fatty acids; plasma palmitic acid concentrations tended to be lower in cows fed HPA, relative to MPA (P = 0.06). Although increasing lecithin did not modify liver enzyme levels, such as sorbitol dehydrogenase, several interactions were observed between palm fat type and lecithin amount but were not of clinical concern. Because lecithin feeding decreased dry matter intake, decreased plasma milk urea nitrogen, and lowered milk de novo fatty acid content, rumen fermentation and biohydrogenation was likely modified.

Lecithin supplementation did not overtly modify total, 16-carbon, or 18-carbon fatty acid intake; however, total fatty acid intake tended to be enhanced by lecithin in cows fed HPA (P = 0.09). Lecithin feeding did not modify apparent dry matter, total fatty acid, or 16-carbon or 18-carbon fatty acid digestibility. An overall effect of lecithin on total, 16-carbon, or 18-carbon absorption was also not observed. Feeding HPA did modify fatty acid intake, digestibility, and absorption, relative to MPA. Specifically, cows fed HPA had greater intakes of 16-carbon and 18-carbon fatty acids (P < 0.05). Feeding HPA markedly reduced total fatty acid and 16-carbon fatty acid digestibility (P < 0.001). Consequently, total fatty acid absorption was lower in cows fed HPA (P = 0.01). The absorption of 16-carbon fatty acid tended to be lower in cows fed MPA (P = 0.09). The MPA diet did include more 18-carbon fatty acids such as oleic and stearic acids, and lower 16-carbon fatty acids, relative to HPA. The inclusion of oleic acid in prilled palm fats has been shown to improve digestibility (de Souza et al., 2018). Collectively, the data affirm that palm fat supplements that are highly enriched in palmitic acid suppressed apparent fatty acid digestibility, and feeding unprotected lecithin, which is susceptible to rumen degradation, does not enhance fatty acid digestibility or absorption in cows fed palm fat. Future research needs to evaluate whether the post-ruminal delivery of lecithin enhances fatty acid digestibility in ruminants.

Lecithin feeding is also a source of the methyl donor choline. Therefore, we explored the ability of lecithin feeding to modulate plasma choline supply. While no effects of lecithin were detected for plasma choline, methionine, or total PC, LPC, or sphingomyelin concentrations, lecithin feeding increased trimethylamine *N*-oxide and dimethyl-glycine, and linearly decreased phosphocholine concentrations ($P < 0.05$). When individual phospholipids were measured using lipidomics, plasma PC and sphingomyelin concentrations increased with lecithin feeding (e.g., PC 35:1 and SM

42:0; $P < 0.05$). Lecithin also increased the ratio of many PC to PE in plasma (e.g., PC 18:0/20:4; $P < 0.05$). These results demonstrate that lecithin-derived choline was degraded in the small intestine to form trimethylamine (to be converted to trimethylamine *N*-oxide in the liver). However, the data would also suggest that some choline may have been absorbed. The observed increase in dimethyl-glycine and individual PC concentrations, lower PC to PE ratios, and reduced phosphocholine concentrations suggests that PC synthesis was upregulated in cows fed lecithin. We cannot conclusively say that an increase in intestinal choline absorption was the cause because increased post-ruminal fatty acids from lecithin degradation could also conceivably enhance phospholipid synthesis in the cow. Nevertheless, the delivery of post-ruminal lecithin as a source of choline deserves further consideration.

Summary

Phospholipids and lysophospholipids are natural emulsifiers of neutral fats. However, the presence of phospholipase and lysophospholipase in the rumen results in their hydrolysis before they can aide in post-ruminal lipid digestion. The ruminal degradation of phospholipids appears to develop with reductions in dry matter intake, modified rumen fermentation, and reduced organic matter and NDF digestibility; albeit these effects are more often observed in cows fed diets low in fiber and enriched in unsaturated oils. The ruminal hydrolytic release and biohydrogenation of unsaturated oleic, and linoleic acids are likely at play. Unfortunately, the current scientific consensus is that feeding unprotected lecithin or lysolecithin fails to improve fatty acid digestibility or absorption. Because bile is enriched in phospholipids (especially PC) and the emulsifying properties of phospholipids have been demonstrated in non-ruminants, it is logical to postulate that the post-ruminal delivery of lecithin or lysolecithin enhances saturated fatty acid digestibility and absorption in the intestine. To make this observation, we must consider the processed form, protection technologies, PC or LPC content, and feeding level of lecithin as well as potential interactions with base diet fatty acid content and composition.

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Using On-Farm Measures to Predict Eating Time and Feed Intake in Dairy Cattle

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Introduction

The modern dairy cow has very high nutrient demands to achieve and maintain the genetic potential for milk production. Intake is one of the most important variables affecting animal performance and as such, is one of the single most significant measures made on a dairy farm (Waldo and Jorgensen, 1981; Roseler et al., 1997). Since intake is highly related to milk production, prediction equations have been created for intake, more specifically dry matter intake (DMI), and one of the most commonly used is the Dairy NRC equation (2001) which includes milk production, body weight, and stage of lactation to predict DMI. Using animal measurements has been the standard approach for creating DMI predictions, but the environment the cow is living in can also play a significant role.

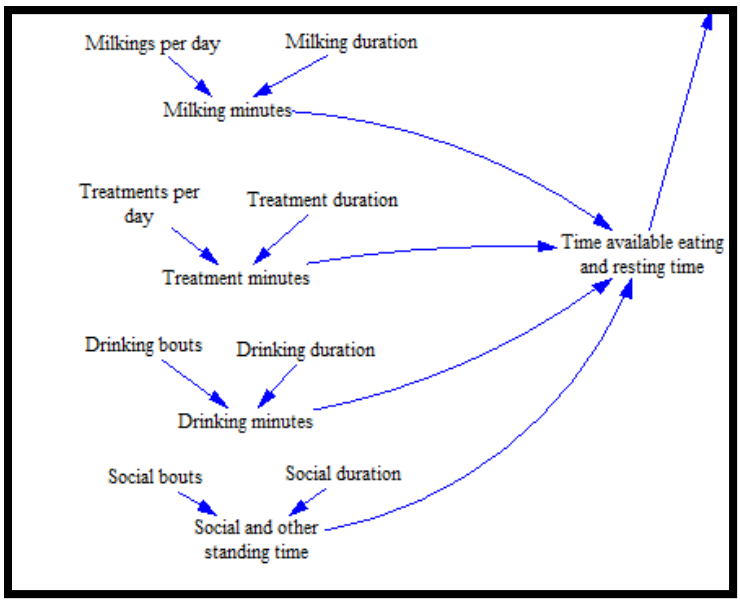

Figure 1. Schematic of time budget for management model.

A major portion of the daily time budget for the modern dairy cow is spent lying down, around 660 to 720 minutes on average, and a second large time requirement is time spent eating, around 300 minutes (Grant, 2004). The management decisions we make, and their consequences, may impede either lying or eating time and can affect DMI such as stocking density, feed frequency, feed availability, lameness, or heat stress (Phillips and Rind, 2001; DeVries et al., 2005; Mantysaari et al., 2006; Cook et al., 2007; Norring et al., 2014; Grant, 2015). Overstocking increases eating rate, displacements at the feed bunk, lameness, and standing time (Grant, 2015). In some studies, increasing the number of daily feedings has increased the time spent eating and decreased lying

time (Phillips and Rind, 2001; DeVries et al., 2005; Mantysaari et al., 2006), whereas lameness has been shown to decrease eating time and increase eating rate (Norring et al., 2014). Heat stress can cause a decrease in DMI, milk yield, and lying time (Cook et al., 2007). These management factors affect the cow's ability to prioritize rest and intake, which is necessary for health and high levels of productivity.

Recently we have focused on trying to quantify and predict the effects of management decisions on DMI using mathematical modeling. The first step was to build the time budget for the dairy cow to include time spent milking, receiving treatments, drinking, and social and other standing time (Figure 1). These were then added up and subtracted from 1440 minutes to get time available for lying and eating. The lying time was calculated by subtracting eating time from the time available for lying and eating (Figure 2). The lying time was then used in the prediction of fat-corrected milk (FCM) to calculate DMI using the Dairy NRC (2001) equation (Figure 2). The weakness of the time budget section is the ability to measure eating and lying time on-farm, and the intake section is dependent on an accurate DMI and milk yield. There is a need to accurately predict eating time, DMI, and milk yield to help increase the accuracy of the management model.

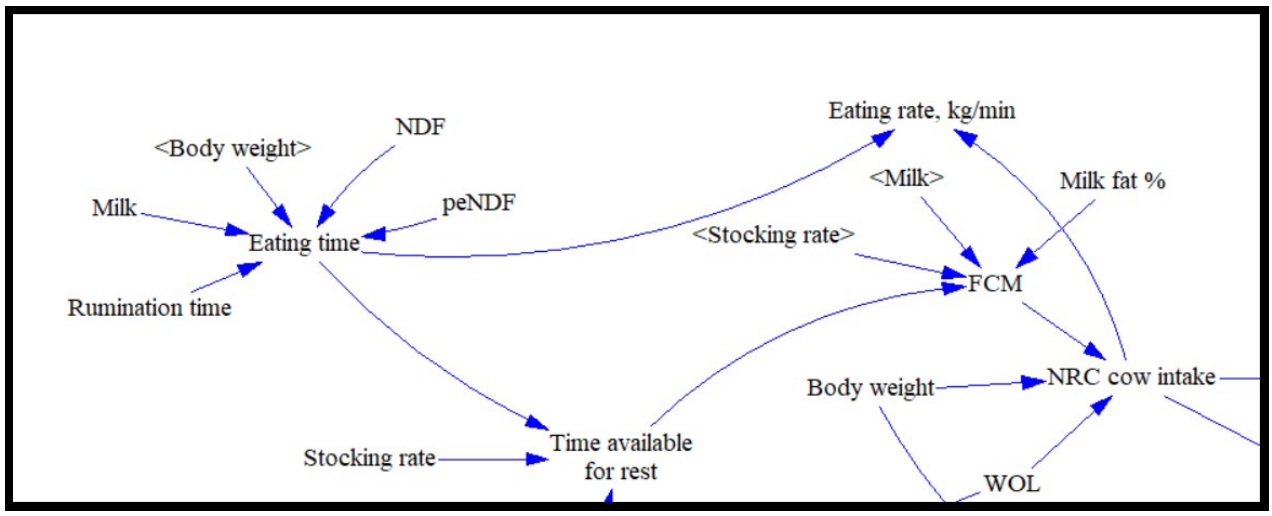

Figure 2. Schematic of eating and resting time and intake layout for management model.

Miner Institute Study:
Predicting Eating Time, DMI and Milk Yield

To begin addressing the issues above, we used 6 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, DMI, and milk yield using common on-farm measures (Kononoff et al., 2003; Cotanch et al., 2014; Miller et al., 2017; Smith et al., 2018; Coons et al., 2019; Miller et al., 2019). The on-farm measures we selected were neutral detergent fiber (NDF) content, physically effective NDF (peNDF), milk yield, body weight, and rumination. We conducted multiple linear

regression (MLR), and partial least squares (PLS) on 20 treatment means from our database. Data were analyzed using Reg and PLS procedures using SAS (version 9.4).

Table 1. Regression statistics for linear prediction equations for eating time, DMI, and milk yield.

Outcome variable	Predictor variable	Partial R-square	Model R-square	VIF
Eating time, min/d	NDF content, % of DM	0.252	0.83	5.5
	peNDF, % of DM	0.045		5.5
	Rumination time, min/d	0.091		4.3
	Body weight, kg	0.069		2.1
	Milk yield, kg/d	0.374		5.4
DMI, kg/d	NDF content, % of DM	0.219	0.69	5.5
	peNDF, % of DM	0.026		5.5
	Rumination time, min/d	0.099		4.3
	Body weight, kg	0.011		2.1
	Milk yield, kg/d	0.334		5.4
Milk yield, kg/d	NDF content, % of DM	0.301	0.82	3.6
	peNDF, % of DM	0.500		3.9
	Rumination time, min/d	0.003		4.1
	Body weight, kg	0.011		1.9

The MLR analysis to predict eating time accounted for 83% of the variance using NDF content, peNDF, rumination time, body weight, and milk yield (Table 1). The MLR analysis to predict DMI accounted for 69% of the variance using NDF content, peNDF, rumination time, body weight, and milk yield. A large proportion of the accounted variance for eating time and DMI was from the milk yield and NDF content with 63% and 55%, respectively. The MLR analysis to predict milk yield accounted for 82% of the variance using NDF content, peNDF, rumination time, and body weight. The NDF content and peNDF accounted for the largest proportion of accounted variance in milk yield with 80%.

Partial Least Squares analysis accounted for 80.1% of the between-treatment variance in eating time, and 4 traits had a variable of importance in projection (VIP) score > 0.9, which included NDF content, peNDF, rumination time, and body weight (Table 2). The PLS analysis accounted for 63.5% of the between-treatment variance in DMI, and 3 traits had a VIP score > 0.9, which included NDF content, peNDF, and milk yield. The PLS analysis accounted for 75.9% of the between-treatment variance in milk yield, and 2 traits had a VIP score > 0.9, which included NDF content and peNDF.

Table 2. Variable of importance in projection (VIP) scores and accounted variance using partial least squares for predicting eating time, DMI, and milk yield.

Outcome variable	Predictor variable	VIP	Accounted variance
Eating time, min/d	NDF content, % of DM	0.92	80.1

	peNDF, % of DM	0.95	
	Rumination time, min/d	1.50	
	Body weight, kg	0.90	
	Milk yield, kg/d	0.46	
DMI, kg/d	NDF content, % of DM	0.92	63.5
	peNDF, % of DM	1.01	
	Rumination time, min/d	0.74	
	Body weight, kg	0.66	
	Milk yield, kg/d	1.47	
Milk yield, kg/d	NDF content, % of DM	1.04	75.9
	peNDF, % of DM	1.52	
	Rumination time, min/d	0.73	
	Body weight, kg	0.29	

Table 3. The summary statistics of 13 published studies with 50 treatments using lactating dairy cows.

Item	Mean	SD	Minimum	Maximum
DMI, kg/d	24.9	2.6	20.5	31.1
Milk yield, kg/d	37.2	6.4	26.2	46.4
Eating time, min/d	228	28	173	318
Rumination time, min/d	469	72	236	564
Body weight, kg	668	47	567	753
NDF content, % of DM	32.6	2.8	27.3	37.5
peNDF, % of DM	25.2	4.6	15.2	34.0

To test the predictive ability of the equations from MLR and PLS we compiled 13 published studies with 50 treatments using lactating dairy cows that included DMI, milk yield, eating time, rumination time, body weight, NDF content and peNDF (Table 3; 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 summary statistics of the studies are presented in Table 3. The mean absolute error (MAE) of eating time, DMI, and milk yield using prediction equations from MLR and PLS using 13 published studies with 50 treatments split into different groups (all, multiparous, primiparous, and mixed) are presented in Table 4. For eating time, the PLS had a lower MAE compared to MLR with the best predictive ability for the multiparous and mixed groups of 35.4 and 35.1 min/d, respectively. Whereas for DMI, the MLR had a lower MAE compared to PLS with the best predictive ability for the primiparous group of 1.4 kg/d. For milk yield, the PLS had a lower MAE compared to MLR with the best predictive ability for the primiparous and mixed group of 4.6 kg/d. The prediction equations can moderately predict eating time, DMI, and milk yield of lactating dairy cows.

Table 4. Mean absolute error (MAE) of eating time, DMI, and milk yield using prediction equations from multiple linear regression (MLR) and partial least squares (PLS) on 13 published studies with 50 treatments.

MAE	Eating time, min/d		DMI, kg/d		Milk yield, kg/d	
Cows	MLR	PLS	MLR	PLS	MLR	PLS
All	57.8	40.2	1.7	2.7	9.1	7.4
Multiparous	56.4	35.4	1.7	3.0	11.2	9.1
Primiparous	100.2	70.3	1.4	2.7	8.4	4.6
Mixed	36.8	35.1	1.6	1.8	3.9	4.6

Summary and Perspectives

This project evaluated the predictive capability of using on-farm measures to predict eating time, DMI, and milk yield to be used in a mathematical model to account for management effects on DMI. We were able to moderately predict using NDF content, peNDF, rumination time, body weight, and milk yield. The greater accuracy of eating time, DMI, and milk yield will increase the sensitivity of the model to management effects. As the development of the model continues, the long term goals will be to build a larger dataset for creating prediction equations, adding additional management decisions to the model, and using on-farm observations to validate the model. Though this is in the early stages of development, there is promise to create a tool to help identify areas of opportunity to optimize the dairy cow's time budget to maximize health and performance.

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Evaluation and development of the Cornell Net Carbohydrate and Protein System v.7 using a unique pasture-based data set

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Introduction

The Cornell Net Carbohydrate and Protein System (CNCPS) has been regularly updated since its first publication in 1992 (Fox et al., 2004, Tylutki et al., 2008, Van Amburgh et al., 2015) and is now widely used for diet formulation in the U.S. with growing usage across the world. Nutritional modelling allows the user to quantify the requirements of an animal and formulate diets, using the available resources, to meet the animal's demands. In the latest CNCPS v.7 (Higgs and Van Amburgh, 2016) key components such as carbohydrate and nitrogen (N) digestion, microbial N (MicN) flow and amino acid (AA) supply have been described in a more dynamic and mechanistic manner. While maintaining the functionality of previous versions, this update provides users with new capabilities and potentially increased precision in diet formulation.

The digestion of protein along the gastrointestinal tract is now calculated on a N basis. By incorporating compartmental analysis, reconciliation across the whole tract can occur, to ensure all N is accounted for throughout each compartment (e.g. rumen, omasum, abomasum, etc.). Within this new structure are mechanistic representations of growth of bacteria and protozoa, including interactions such as protozoal predation of bacteria. Rather than accounting for protozoa statically, by reducing the theoretical maximum growth yield of bacteria from 0.5 to 0.4 g cells per g carbohydrate fermented as in previous versions (Russell et al., 1992), the influence of protozoa on nutrient digestion and microbial flow is now described mechanistically and dynamically. This has the potential to predict a more precise quantification of metabolizable AA supply to the animal, as protozoa have been show to contribute 5-23% of MicN flow (Sylvester et al., 2005, Fessenden et al., 2019). Further, the composition of protozoa is different than bacteria, especially for certain AA such as lysine (Jensen et al., 2006; Fessenden et al, 2019). Finally, protozoa have also been implicated in altering the rumen environment such as ammonia N production and pH regulation (Jouany et al., 1988, Williams and Coleman, 1988, Hristov and Jouany, 2005).

Utilizing literature data sets, evaluations of v.7 indicated a strong ability to predict non-ammonia N (NAN) flow at the omasal canal (Higgs and Van Amburgh, 2016). However, within this NAN flow, biases were present where non-ammonia, non-microbial N (NANMN) flow was over predicted and the MicN flow was under predict compared to the observed literature values. While literature data sets are a powerful tool to evaluate models, in many of these studies protozoal flow was not directly measured due to the

difficulty of protozoal isolation. Thus, the NANMN fraction reported might have included protozoal N and conversely, the MicN pool measured might not be accounting for this protozoal N (Brito and Broderick, 2007). To more fully evaluate these constraints on model development, our laboratory conducted an omasal flow study, incorporating a rapid technique to isolate mixed protozoa in order to directly measure protozoal flow (Fessenden et al., 2019). While the total MicN flow was predicted accurately in the study, the model underestimated protozoal flow by approximately 43%. This evaluation suggested that more studies directly measuring protozoal N flow and its contribution to the total MicN flow are required in order to better describe the contributions of protozoa to total microbial flow and the interaction among protozoa and bacteria. Further, it was important to study this in a feeding management system different from the data sets used to develop the model and to have data outside what is available in the literature and Northeast U.S feeding systems.

The CNCPS was developed with data utilizing corn silage and alfalfa based diets with subsequent model evaluations being performed on similar data sets. In vitro and in vivo analysis suggests that fresh perennial ryegrass (PRG) swards, managed intensively, are rapidly degradable with a large proportion of the aNDFom in the potentially digestible pool (~90%); drastically different to conventional forages used in the U.S. Further, a large proportion of the feed N in this type of pasture is digested in the rumen (Sairanen et al., 2005) contributing poorly to metabolizable protein supply. These feed behaviors, that are distinctly different from typical U.S. diets, have the potential to provide a boundary test to challenge the robustness of the underlying biology and feed fractionation schemes of the CNCPS. Therefore, we designed an experiment incorporating pasture-based diets, rapid isolation of mixed protozoa, and the omasal sampling technique to generate a unique data set for model evaluation and development.

Omasal Flow Experiment

In temperate regions, pasture-based diets are an important source of nutrients for the production of animal products and are an appropriate and beneficial use of the resource (Dijkstra et al., 2013). Whilst well-managed pasture is highly digestible, energy intake is typically reported as first limiting milk solids production. There is a large amount of research investigating the effect of providing energy dense supplements to grazing dairy cattle however, wide variation in milk response, dry matter intake (DMI) and substitution effects exist with little explanation of how or why different responses to these supplements occur (Bargo et al., 2003). In this experiment, we utilized rolled barley (RB) as a supplement and evaluated its effects on milk production, rumen metabolism, rumen digestion kinetics and omasal flow of nutrients in lactating dairy cattle fed fresh PRG indoors. We also quantified the rumen pool size and omasal flow of bacteria and protozoa. As RB is a source of rapidly degrading starch, we hypothesized that it would stimulate protozoal growth which would provide treatment effects for model parameter evaluation (Chamberlain et al., 1985, Jaakkola and Huhtanen, 1993, Ahvenjärvi et al., 2002).

This study was undertaken at Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy Co. Cork, Ireland. Ten ruminally cannulated Holstein cows averaging 49±23 DIM and 513 ± 36 kg of BW were assigned to one of two treatments in a switchback design. Treatments were (on a DM basis) 100% PRG (**G**) or 80% PRG and 20% RB grain (**G+RB**). Swards of PRG were mechanically cut twice daily using a "zero grazer" process where the grass is cut at 4 cm above ground level with no additional processing so the forage is provided as is and can be 20 to 30 cm long. The forage was fed across 6 meals daily indoors with RB grain being fed at milking as 2 equal meals. Refusals of both PRG and RB were collected and weights recorded with feeding rate being adjusted daily to yield refusals of 5% to 10% of daily intake. Daily samples of PRG and RB were dried at 105 °C and analyzed for DM. Additional daily samples were either freeze dried or oven dried at 60 °C before being ground and analyzed for chemical composition using wet chemical methods (Table 1). The trial consisted of three 29 d periods where each period consisted of 21 d of diet adaptation/wash-out and 8 d of marker infusion and animal sampling. During this latter phase a double marker system utilizing CoEDTA (Udén et al., 1980) and undegraded NDFom (uNDFom; Raffrenato et al., 2018) was used to quantify liquid and particle flow at the omasal canal, respectively. Ytterbium was used as part of a triple-marker system, but was abandon upon analysis and will not be discussed. Ytterbium recovery in the feces was low indicating the Yb didn't bind at a high rate or concentration. This further indicates that due to the rate and extent of fiber digestion in the rumen and rate of passage out of the rumen of both the fiber and marker, there was low affinity and binding, which led to a failure of the marker. Additionally, double-labelled ammonia sulfite ($^{15}N^{15}N$-ammonia sulfite, 10% enriched $^{15}NH_4SO_4$) was continuously added to the rumen in order to quantify microbial flow and pool size.

Samples of whole omasal contents were collected from the omasal canal using the sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). The pattern of sampling was in three 8 hour intervals: at 16:00, 18:00, 20:00, and 22:00 h on day 24; at 00:00, 02:00, 04:00, and 06:00 h on day 26; and at 08:00, 10:00, 12:00, and 14:00 on day 27. Sample times were chosen to encompass every two hours of the average twenty-four hour cycle. Fecal grab samples were obtained following the same sampling pattern. At the end of each session, bacterial isolations were performed according to Whitehouse et al. (1994) with modifications. In tandem, an additional 250-mL sample was obtained and immediately processed to isolate protozoa using flocculation and filtration techniques, as described in Fessenden et al. (2019). On day 28 and 29 of each period, rumen contents were evacuated, weighed, mixed, and a representative sample was obtained and stored at −20°C. Rumen contents were returned to the cow via the rumen cannula within 30 min of evacuation. All data were analyzed with a mixed-effects model, using fixed effects of sequence, period, treatment, interaction of period and treatment and the random effect of cow within sequence. For the purposes of this paper, the fixed effect of treatment will be discussed.

Results and Discussion

Diet nutrient composition

Crude protein content of the harvested PRG was slightly lower than anticipated, averaging 16.3% across the three experimental periods (Table 1). Typically, mid-season pastures are approximately 18% crude protein but this can be extremely variable depending on factors such as climatic conditions and N fertilizer application (Peyraud and Astigarraga, 1998). A 12-hour in vitro fermentation time point was included in the analysis of the PRG aNDFom digestibility, along with the 30, 120 and 240 h time points as described by Raffrenato et al. (2019). Given the rapid digestion of the PRG, the 30 h measurement misses a significant portion of the rapidly digestible aNDFom, therefore, to analyze this grass, we needed to include a 12-hour time point to better describe the degradation curve of intensively managed PRG swards (Dineen et al., unpublished). Output from the rate calculations of Raffrenato et al. (2019) partitioned 80%, 20% and 9% of the aNDFom into the fast, slow and indigestible pools with rates of 14% h^{-1}, 3% h^{-1} and 0% h^{-1}, respectively. Crude protein content, water-soluble carbohydrate (WSC) and aNDFom content were all numerically lower in G+RB diets compared to G diets (Table 1). Starch content, as was intended in diet formulation, was greater with supplementation. This resulted in an increase of non-fiber carbohydrate (NFC) for the G+RB diets however, the high WSC content of PRG prevented a drastic difference in the NFC content between diets. The content of indigestible aNDFom in the RB supplement was increased compared to PRG (33.0 vs. 9.9% uNDFom, % of aNDFom) which seems to be due to the hull material being included in the barley supplement.

Animal performance and rumen characteristics

During the milk sampling phase (day 21-23; Table 2), total DMI tended to increase in G+RB diets compared with G ($P = 0.11$). This was achieved through consumption of the RB offered in substitution for 0.88 kg of pasture DM per kg of RB DM consumed. This is similar to the results observed by Delagarde and Peyraud (1995) who fed comparable diets. The inclusion of RB had no effect on daily milk yield, ECM or milk solids (kg fat + protein). However, this study was not specifically designed to assess effects on milk production. Milk fat content decreased in cows fed the G+RB diet, whereas milk protein content increased which are similar to the results observed in the review by Bargo et al. (2003) of studies providing energy dense supplements to pasture-based diets. Milk urea N was lower in G+RB diets compared with G (12.7 vs. 16.5 mg/dL; $P < 0.01$) which might be explained by reduced ruminal ammonia pool sizes and concentration in G+RB cows (Table 3). This might have occurred due to the increased incorporation of feed N into MicN in G+RB cows as indicated by the higher MicN flow, discussed further below (Table 6). Feed efficiency (ECM/DMI) was reduced in G+RB diets compared with G (1.36 vs. 1.45; $P < 0.05$) and this seems surprising given the added fermentable carbohydrate. Concentrations of total VFA, propionate, valerate and isovalerate all increased due to RB supplementation (Table 3). Reticulum pH, measured using eCow® boluses (Devon, U.K) were not different among treatments, averaging 6.36 and 6.37 for diets G and G+RB,

respectively. These means were slightly higher than the mean reported by Kolver and deVeth (2002) of 6.15 for a number of pasture-based treatments.

Table 1. Nutrient composition (mean ± SD)[1] of experimental diets and selected supplement used in the experiment

	Diet[2]		
Nutrient composition[4]	G	G+RB	RB[3]
DM, %	21.0 ± 3.0	34.7 ± 3.6	86.9 ± 0.8
CP, % of DM	16.3 ± 3.1	15.4 ± 2.7	11.6 ± 0.4
Soluble protein, % of CP	35.3 ± 3.0	31.5 ± 2.9	17.1 ± 1.9
Starch, % of DM	2.2 ± 0.5	14.4 ± 1.5	60.7 ± 0.7
Sugars (water soluble), % of DM	23.9 ± 1.6	19.3 ± 1.1	1.9 ± 0.2
NFC, % of DM	37.7 ± 3.8	43.5 ± 3.0	65.6 ± 2.7
aNDFom, % of DM	36.3 ± 1.2	32.7 ± 1.5	19.2 ± 1.0
12-h uNDFom, % of aNDFom	50.9 ± 8.5	-	71.0 ± 0.3
30-h uNDFom, % of aNDFom	20.9 ± 2.8	-	-
72-h uNDFom, % of aNDFom	-	-	38.5 ± 1.4
120-h uNDFom, % of aNDFom	11.8 ± 0.3	-	33.0 ± 0.6
240-h uNDFom, % of aNDFom	9.9 ± 0.4	-	-
ADF, % of DM	20.7 ± 1.7	17.5 ± 1.9	5.0 ± 0.6
ADL, % of NDF	4.2 ± 0.8	5.8 ± 0.9	11.8 ± 2.7
Ether extract, % of DM	3.1 ± 0.5	2.9 ± 0.4	1.7 ± 0.2
Ash, % of DM	6.6 ± 0.5	5.6 ± 0.4	2.6 ± 0.6

[1]Analyzed values from 12 samples (4 day x 3 period).
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]RB = rolled barley grain.
[4]NFC = non fiber carbohydrate; aNDFom = amylase- and sodium sulfite-treated NDF corrected for ash residue; uNDFom = undigested amylase- and sodium sulfite treated NDF corrected for ash residue; ADF = acid detergent fiber; ADL = acid detergent lignin.

Table 2. Effect of rolled barley inclusion on dry matter intake (DMI), milk production, and animal performance of pasture-fed lactating dairy cattle

	Treatment[2]			
Item[1]	G	G+RB	SEM	*P*-Value
DMI, kg/d	17.2	17.6	0.3	0.11
Milk yield, kg/d	21.2	21.4	1.0	0.81
ECM, kg/d	24.6	24.1	0.8	0.70
Milk solids[3], kg/d	1.68	1.65	0.05	0.64
Milk fat, %	4.52	4.28	0.16	<0.05
Milk fat, kg/d	0.96	0.90	0.03	0.09
Milk crude protein, %	3.44	3.54	0.07	<0.05
Milk crude protein, kg/d	0.73	0.75	0.02	0.19
MUN[4], mg/dL	16.5	12.7	0.9	<0.01
Feed efficiency[5]	1.45	1.36	0.05	<0.05
BW change, kg/d	7.8	6.6	4.2	0.85

[1]Values calculated from data collected on d 21 to 23 of each experimental period.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.

[3]Milk solids = kg fat + protein
[4]MUN = milk urea nitrogen.
[5]ECM/DMI.

Digestion of DM, OM and aNDFom

As the experimental animals were exposed to increased human contact during the omasal sampling procedure, which might have slightly reduced their DMI, separate intakes are reported for the milk production data versus the omasal sampling data (Tables 2 and 4, respectively). During the sampling phase (day 24-28), the inclusion of RB increased DM and OM intake in comparison to G diets, while flow of DM and OM measured at the omasal canal were also increased ($P < 0.01$). The amount of DM truly degraded in the rumen tended to be greater in G+RB diets ($P = 0.13$). Organic matter truly degraded in the rumen increased ($P < 0.01$) in cows fed the G+RB diet. Compared with G diets, the inclusion of RB reduced the total tract digestibility of DM and OM that was consumed ($P < 0.01$).

Table 3. Effect of rolled barley inclusion on rumen concentration and pool size[1] of ammonia N, VFA and reticulum pH

	Treatment[2]			
Item	G	G+RB	SEM	*P*-Value
Ammonia N pool size, g	6.4	3.9	0.5	<0.01
Ammonia N concentration, mg/dL	9.0	5.9	0.5	<0.01
VFA[3] concentration, mM				
Total VFA	121.8	126.0	2.0	<0.05
Acetate	75.8	74.6	1.1	0.32
Propionate	25.7	30.2	0.8	<0.01
Butyrate	16.0	16.2	0.3	0.67
Isobutyrate	0.9	0.8	0.1	0.43
Valerate	1.7	2.4	0.2	<0.01
Isovalerate	1.6	1.8	0.1	<0.05
Reticulum pH	6.36	6.37	0.2	0.78

[1]Nutrient concentration × rumen liquid volume measured from total rumen evacuation.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]VFA = volatile fatty acid

The intake of aNDFom was reduced in cows fed the G+RB diets; however, aNDFom flow at the omasal canal was increased, relative to cows fed the G diet. Accordingly, aNDFom digestibility decreased, both ruminally and total tract, in cows fed the G+RB diet. In addition, rumen pool size of aNDFom and uNDFom increased ($P < 0.05$; data not presented) due to supplementation of RB. Low rumen pH is typically cited as the cause for reduced aNDFom digestibility. However, in the present study low ruminal pH cannot be linked to the decreased aNDFom digestibility, as the rumen pH was not different among treatments, averaging 6.36. Further, de Veth and Kolver (2001) reported that in vivo digestibility of pasture might not be compromised by low ruminal pH (< 6.0) for dairy cows fed diets of high quality pasture. Reduced aNDFom digestibility can be a

multifaceted issue. The concentration of uNDF was higher in the RB grain compared with PRG, as discussed earlier, due to the grain containing hull material. This might have contributed to the reduction in aNDFom digestibility, as reported in other studies (Van Vuuren et al., 1993, Sairanen et al., 2005). Additionally, in this experiment, barley starch altered rumen metabolism with higher propionate concentrations being observed and this change in the type of carbohydrate digested might have created a potential deficiency. In a review by Hoover (1986), the author suggested that rumen ammonia N concentrations required to optimize nutrient digestion was 6.2 mg/dL while microbial growth was optimized at a lower ammonia N concentration of 3.3 mg/dL. Other authors speculated that the rumen ammonia N concentration required by the particulate associated microbes digesting fiber might be greater than that of the fluid associated microbes (Allison, 1980, McAllan and Smith, 1983). Further, Satter and Slyter (1974) demonstrated that a rumen ammonia level of 5 mg/dL was the minimum required to maintain adequate microbial growth. In the current experiment, rumen ammonia N concentration was close to the threshold of 5.0 mg/dL in cows fed the G+RB diet, potentially explaining a further portion of the reduced aNDFom digestibility. This suggests that on a dynamic basis, with the rumen ammonia levels most likely variable throughout the day, at times the NFC bacteria outcompete the fiber bacteria for ammonia, decreasing aNDFom digestion.

Fiber digestion is predicted in v.7 of the CNCPS utilizing 1) the uNDFom240 assay (Raffrenato et al., 2018) to determine aNDFom available for microbial degradation 2) fractionation of this calculated pdNDFom into two digestible pools that degrade concurrently but at differing rates (Raffrenato et al., 2019). In the present study, ruminal aNDFom digestion was predicted well in comparison to observed for both the G diet (4,326 v 4,218 g day^{-1}, respectively) and the G+RB diet (3,965 v 3,540 g day^{-1}, respectively). The means of period 2 and 3 for the G diet can be used to remove the variation caused by low ruminal ammonia N concentration due to low forage N content and the associative effect of RB in Period 1. Accordingly, the difference between predicted and observed for ruminal aNDFom digestion was 1.6% (Period 2) and 1.1% (Period 3) above the measured amount. These results indicate that the in vitro approach used to calculate pools and rates, in combination with model predicted passage rates, accurately describe in vivo observations of ruminal aNDFom digestion in animals fed high quality pasture.

Table 4. Effect of rolled barley inclusion on digestibility of DM, OM, and aNDFom

Item[1]	Treatment[2] G	Treatment[2] G+RB	SEM	*P*-Value
DM				
Intake, kg/d	16.1	17.1	0.4	<0.01
Flow at omasal canal, kg/d	10.5	11.3	0.5	<0.01
Apparently digested in the rumen, kg/d	5.6	5.8	0.2	0.41
Truly digested in the rumen,[3] kg/d	12.1	12.7	0.3	0.13
% of DMI	74.3	74.2	2.1	0.94
Total-tract apparent digestibility, %	82.8	79.7	0.3	<0.01
OM				
Intake, kg/d	15.1	16.1	0.4	<0.01
Flow at omasal canal, kg/d	6.9	7.7	0.3	<0.01
Apparently digested in the rumen, kg/d	8.2	8.4	0.2	0.27
Truly digested in the rumen, kg/d	13.2	13.9	0.3	<0.01
% of OM intake	87.9	86.1	0.6	<0.01
Total-tract apparent digestibility, %	85.2	82.0	0.3	<0.01
aNDFom				
Intake, kg/d	5.8	5.6	0.2	<0.05
Flow at omasal canal, kg/d	1.6	2.0	0.1	<0.01
Apparently digested in the rumen, kg/d	4.2	3.6	0.1	<0.01
% of aNDFom intake	72.3	63.1	0.9	<0.01
% of pdNDFom[4] intake	80.4	72.3	1.0	<0.01
Total-tract apparent digestibility, %				
% of aNDFom intake	83.2	74.5	0.6	<0.01
% of pdNDFom intake	92.5	85.4	0.7	<0.01

[1]Values calculated from data collected on d 24 to 28 of each experimental period.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[3]Corrected for microbial and VFA contribution to flows.
[4]pdNDFom = potentially digestible aNDFom.

Nitrogen Flow

Nitrogen intake was similar across treatments (Table 5). However, compared with G diets the inclusion of RB increased the flow of NAN at the omasal canal ($P < 0.01$). This is consistent with the results observed by Van Vuuren et al., (1993) that offered a starch supplement and Sairanen et al. (2005) that offered a low CP pelleted supplement. While both Younge et al. (2004) and O'Mara et al. (1997) reported no difference in NAN flow, values observed in both studies were increased for supplemented diets compared to non-supplemented diets. In the current experiment, the increase in NAN can be attributed to the increased flow of MicN in G+RB diets compared with G ($P < 0.01$). There was no difference in NANMN flow between the treatments however, the contribution of NANMN to the total NAN flow was relatively low (12%) compared to previous studies (O'Mara et al., 1997, Younge et al., 2004). The NANMN flow is typically estimated by difference (i.e. NAN flow – MicN flow) therefore, any error in either of these estimations will be partitioned into the NANMN flow. A key difference between studies was that in Younge et al. (2004)

and O'Mara et al. (1997), purine derivatives were utilized to determine MicN which have been shown to have lower precision and accuracy compared with techniques using ^{15}N, used in the current study, while also underestimating MicN flow (Klopfenstein et al., 2001, Firkins and Reynolds, 2005, Reynal et al., 2005, Ipharraguerre et al., 2007). Further, Sairanen et al. (2005) reported that the purine derivative method underestimated MicN flow in the pasture only treatment by 15% and thus, over predicting the NANMN flow by the same amount. This inaccuracy has further implications in regards to the determination of true ruminal digestible N, as an underestimated MicN flow will underestimate true digestibility. In the present study, the average true ruminal digestible N was 88%, was not different between treatments and was comparable to the 85% reported by Sairanen et al. (2005). Of the total MicN flow, protozoal N contributed on average 22% and was not different between treatments. There are few data describing protozoal flow in pasture-fed cows however, this average was within the range of that proposed by Dijkstra et al. (1998; 10.7 – 26.1%) in computer simulations of animals consuming similar DMI. In contrast to our hypothesis, supplementation with RB did not increase protozoal N flow. It is difficult to ascertain the reason for this however; the high WSC content of the fresh temperate PRG might have provided ample sugar to sustain high protozoal growth (Clarke, 1965, Williams and Coleman, 1988). Further, recent studies have clearly demonstrated that mixed protozoa can sequester sugar away from bacteria, giving protozoa a competitive advantage and stabilizing fermentation in the rumen (Denton et al., 2015). As the majority of N in high quality pasture is ruminally digestible (> 80%), this data describes the significant dependence, of animals grazing such swards, for MicN as their main source of metabolizabe AA. Thus, it is essential to maintain optimum rumen environments with ample supply of fermentable material to achieve desired animal performance from high forage diets.

Microbial dynamics

In the CNCPS, microbial growth is described based on the amount and type of carbohydrate fermented, as this is the main source of energy for microorganisms (Russell et al., 1992). Models designed to calculate microbial yield based on organic matter digestion, ignores the fact that most ruminal bacteria are unable to utilize protein, fat or lipid as an energy source for growth (Nocek and Russell, 1988). Compared with G diets, the inclusion of RB increased both the pool of rumen fermentable carbohydrates ($P < 0.01$) and the true ruminal carbohydrate digestion rates ($P < 0.01$; Table 6). This is consistent with the observed increase in MicN flow for G+RB diets (Table 5). Rumen microbial OM pool was not different among treatments, and averaged 24% of the rumen OM pool which is similar to results previously reported (Craig et al., 1987, Fessenden et al., 2019). Rumen protozoal N pool similarly was not affected by treatment, however; protozoa contributed considerably less to the total MicN pool in the rumen (6%) in comparison to at the omasal canal (22%). Sylvester et al. (2005) reported similar protozoal proportions in the rumen (9%) using a real-time polymerase chain reaction assay.

Table 5. Effect of rolled barley inclusion on the flow of nitrogen in pasture-fed lactating dairy cattle

Item[1]	Treatment[2]		SEM	*P*-Value
	GO	G+RB		
N intake, g/d	429	424	11	0.53
Flow at omasal canal				
Total N, g/d	394	438	18	<0.01
Ammonia N, g/d	21	14	1	<0.01
NAN				
g/d	373	422	18	<0.01
% of N intake	90.8	99.3	2.8	<0.05
NANMN				
g/d	49.1	47.7	4.1	0.78
% of N intake	11.6	11.0	0.9	0.65
Microbial NAN				
g/d	324	374	15	<0.01
% of total NAN	87.1	88.8	0.8	0.17
Bacteria NAN				
g/d	248	298	18	<0.01
% of microbial NAN flow	76.5	80.1	3.2	0.23
Protozoa NAN				
g/d	79	73	11	0.55
% of microbial NAN flow	23.5	20.0	3.2	0.23

[1]N = nitrogen; NAN = non-ammonia nitrogen; NANMN = non-ammonia, non-microbial nitrogen.
[2]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.

Fractional growth rate of bacteria tended to increase in cows feed the G+RB diet, with a number of studies reporting similar effects (P = 0.07; Nocek and Russell, 1988). While fractional growth rate of protozoa was similar between diets, the average observed (0.35 h^{-1}) is extremely high in comparison to current assumptions of the theoretical maximal fractional growth rate. Unfortunately, data in this area are lacking also (Firkins and Yu, 2006), as many in vivo studies investigating specific microbial outflows do not measure the rumen pool size and hence, cannot directly determine fractional growth rate. This limits our ability to compare the current observed result to previous literature. However, as noted by Wells and Russell (1996), the observed growth rate of rumen microbes does not address turnover. The true growth rate can be calculated as; observed growth rate/(1-turnover). Microbial turnover constants as high as 90% have been reported (Firkins et al., 1992) with ruminal dilution rate cited as a key factor influencing this variable (Wells and Russell, 1996). In the present study, the fluid passage rate averaged 0.21 h^{-1}, and because protozoa have predominantly been shown to associate with the liquid phase (Hungate, 1966, Dehority, 1998), this provides a mechanism to explain protozoal growth efficiency. Sylvester et al. (2009) demonstrated that rumen ciliated protozoa can decrease generation time in response to increasing dilution rate; Harrison et al. (1976) reported a similar effect. Further, it should be recognized that the rumen of a grazing cow seems optimal for efficient protozoal growth due to an ample supply of sugars, soluble true protein and moderate pH levels across the day (Clarke, 1965, Williams and Coleman, 1988). The reciprocal of dilution rate determines the fluid retention time, which averaged

5 h in this experiment. Thus, for the protozoa to be associated with the fluid, a generation time of less than 5 h is required to maintain viable rumen populations (Dehority, 2003). Protozoal generation time was not affected by treatment and averaged 4 h in the current experiment. To the author's knowledge, a generation time this short in the rumen has only once been previously reported (Warner, 1962). The fresh PRG has a high digestion rate, and in the case of this study, particle size was supplied to the cattle at 20 to 30 cm, and the rumen turnover was also quite high averaging about 0.125 per h. That means the carbohydrate turns over about every 8 hours in these pasture fed cattle, thus even if a portion of the protozoa are "particle" associated, they still need to have a generation interval that is faster than previously characterized other than that reported by Warner (1962). Further studies are required to confirm the protozoal growth rate and efficiency observed in this study.

The observed Yg (yield of microbial DM per gram of carbohydrate degraded) increased in cows fed the G+RB diets compared with G (0.65 vs. 0.54, respectively). Variable Yg values, in vitro, have previously been reported due to differing carbohydrate sources (Nocek, 1988) however, values greater than 0.5, the theoretical maximum (Isaacson et al., 1975) are rare. This maximum calculated by Isaacson et al. (1975) and those measured for individual species (Russell and Baldwin, 1979, Theodorou and France, 2005) are often determined in pure cultures or in vitro environments. Due to the complexity of replicating in vivo conditions, it is possible that microbial yields reported in vitro might be depressed. Stouthamer (1973) reported, using a biochemical approach, a maximal Yg of approximately 0.8 g/g of glucose, indicating the potential for higher yields to be achieved in vivo. Again, these pasture diets are providing readily available and highly digestible carbohydrates that support the concept of faster growth rates as the whole of rumen contents turn over much faster than any traditional North eastern U.S. diet.

CNCPS v.7 predicted versus observed nitrogen flows

To evaluate the capacity of the CNCPS v.7 to predict N flows at the omasal canal, in pasture-fed dairy cows, model predicted estimates were compared against that of measured in the current experiment. The NAN flow predicted was in good agreement with observed (363 vs. 397 g N day^{-1}, respectively), a 9% underestimation. However, the biases reported in both the evaluations of Higgs and Van Amburgh (2016) and that of Van Amburgh et al. (2015; CNCPS v. 6.5) were present in the current evaluation. The MicN flow was under predicted compared to observed (246 vs. 349 g N day^{-1}, respectively) while NANMN flow was considerably over predicted (117 vs. 48 g N day^{-1}, respectively). The under prediction of MicN flow seems to particularly stem from a reduced bacterial N flow. The underestimation of protozoal flow in the current evaluation was less severe than that of Fessenden et al. (2019; 22% vs. 43%), potentially due to the high WSC content of the diet driving protozoal growth. Consequently, this large protozoal population increases the quantity of bacterial N predated by the protozoa, contributing to the reduced MicN flow. This provides further justification to update the growth rate and passage of protozoa, which are currently associated in the particle phase, to be in the liquid phase, within the structure of v.7. Further, the assumptions that protozoa retain only 50% of the N

consumed (Williams and Coleman, 1988) and a growth rate of half the fractional carbohydrate degradation rate seems too drastic especially under the current experimental condition of rapid protozoal generation times. There are a few potential offsets around all of these predictions of protozoal predation of bacteria, feed protein degradation and the high rate of passage of the liquid phase that all interact to provide part of the MP supply. For example, the current rate of degradation of the B1 protein pool is 15%/h for pasture, which might be too slow given the microbial growth rates and the degradation rate of the fast pool of aNDFom. However, accurate in vivo rates of N degradation are very difficult to quantify in vitro, thus further omasal flow measurements might be required.

Table 6. Effect of rolled barley inclusion on rumen pool sizes, fractional rates of microbial growth and nutrient digestion and generation time

Item	Treatment[1]		SEM	*P*-Value
	GO	G+RB		
Rumen pool size				
Digestible OM,[2] kg	4.75	5.22	0.35	0.07
Total fermentable CHO,[3] kg	3.58	4.19	0.30	<0.01
Total NAN, g	276	289	12	0.15
Microbial NAN, g	199	208	9	0.29
Microbial OM proportion of rumen OM pool, %	24.5	23.8	0.7	0.37
Bacteria NAN,[4] g	186	196	10	0.23
Protozoa NAN, g	13	12	3	0.73
Protozoa NAN pool, % total microbial NAN pool	6.6	5.9	1.4	0.69
Rumen kinetics				
Fractional growth rate of bacteria,[5] h^{-1}	0.056	0.064	0.003	0.07
Fractional growth rate of protozoa,[5] h^{-1}	0.412	0.286	0.084	0.22
Fractional growth rate of all microbes, h^{-1}	0.069	0.076	0.003	0.09
Ruminal true OM digestion rate, g/h	551	580	13	<0.01
Ruminal true CHO digestion rate, g/h	453	479	11	<0.01
Fractional rate of OM digestion,[6] h^{-1}	0.120	0.115	0.006	0.33
Fractional rate of CHO digestion,[6] h^{-1}	0.133	0.121	0.008	<0.05
Observed Yg,[7] g of cells / g of CHO degraded	0.54	0.64	0.04	<0.01
Generation time of bacteria,[8] h	18.8	16.6	0.9	0.10
Generation time of protozoa,[8] h	3.8	4.1	0.5	0.65
Generation time of microbes,[8] h	15.2	13.9	0.7	0.14
Fluid retention time,[9] h	5.0	5.1	0.2	0.71

[1]G = 100% (DM basis) perennial ryegrass; G+RB = 80% perennial ryegrass and 20% rolled barley grain.
[2]Measured OM from rumen evacuation, corrected for microbial OM and undigested NDF after 240 h of in vitro digestion and analyzed with amylase, sodium sulfite and ash corrected (Raffrenato et al., 2018).
[3]Rumen OM pool – (rumen CP pool – microbial CP pool) – (rumen DM pool × diet fat content).
[4]Microbial NAN pool – protozoal NAN pool

[5]Bacterial or protozoal daily flow (g/h)/bacterial or protozoal pool size (g)
[6]Organic matter or carbohydrate degraded (g/h)/ organic matter or carbohydrate rumen pool size (g)
[7]Fractional microbial growth rate/fractional rate of CHO digestion.
[8]Reciprocal of fractional growth rate of bacteria, protozoa or all microbes
[9]Reciprocal of fluid dilution rate

Conclusions

The inclusion of RB into pasture-based diets in the current study increased DMI, rumen pool size of fermentable carbohydrate and the rate of carbohydrate degradation. However, G+RB diets decreased total tract digestibility of DM, OM and aNDFom. Additionally, the NAN flow at the omasal canal increased because of increased MicN flow (50 g), in G+RB diets compared to G. The average contribution of MicN to the total flow of NAN together with high ruminal digestibility of feed protein portrays the large dependence of pasture-fed cattle on microbial protein supply. Although animals grazing pasture-based diets are often cited as being energy first limited, the increased performance typically achieved by supplying energy dense supplements might be through the mechanism of a rise in MicN flow and hence increased metabolizable AA supply – provided adequate rumen N is available. Further research is required to disentangle the mechanisms of increased milk solid production when energy dense supplements are fed as the responses are variable suggesting other limitations under certain conditions.

Evaluation of the capacity of CNCPS v7 to predict NDF degradation in vivo, from in vitro analysis and mathematical modeling, indicates the high precision of this approach. Further refinement is required, to capture the interacting effects of NFC and low rumen ammonia N concentrations on in vivo aNDFom digestion in pasture-fed animals. Finally, although NAN flow at the omasal canal was predicted well, potential modifications have been described to reduce the biases in MicN and NANMN flow predictions.

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Choline and Methionine for Transition Dairy Cows – How Interchangeable Are They?

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Introduction

Choline and methionine both have essential roles in mammalian metabolism. Choline is a quasi-vitamin that has a variety of functions, including that as the predominant phospholipid contained in the membranes of all cells in the body (as phosphatidylcholine), a component of the neurotransmitter acetylcholine, and as a direct precursor to betaine in methyl metabolism (Overton and Waldron, 2004). Furthermore, choline deficiency in monogastric species results classically in fatty liver development among other symptoms.

Methionine is an essential amino acid and building block for protein and typically considered one of the two most limiting amino acids for production of milk and milk protein in lactating dairy cows (National Research Council, 2001). In addition, the roles of methionine in regulating metabolic processes as well as innate immunity and oxidative metabolism continue to be elucidated (Martinez et al., 2017). Methionine can contribute to biosynthesis of phosphatidylcholine through its role as a methyl donor and, in a study conducted a number of years ago using lactating goats and radiolabeled choline and methionine to determine the kinetics and interconversions between the two compounds, 6% of the choline pool was derived from methionine (Emmanuel and Kennelly, 1984).

Choline and methionine each have been the focus of a number of studies involving transition cows during the past 20 years and, as detailed below, both have demonstrated positive effects on cow productivity during early lactation. Given the interrelationships described above, questions are often asked regarding the potential interchangeability of these nutrients. In this paper, I will review the research conducted for both of these nutrients in the transition dairy cow as well as discuss the potential for interchangeability of these nutrients in transition cow metabolism.

Choline Supplementation During the Transition Period

Piepenbrink and Overton (2003) determined that cows fed rumen-protected choline (RPC) during the precalving period and continuing through early lactation tended to have increased fat-corrected milk yields (average response + 5.3 lbs/d) during early lactation along with a trend for decreased storage of radiolabeled palmitate as liver triglycerides in vitro and increased concentrations of liver glycogen, implying improved liver metabolism. Diets in this study were formulated to meet grams per day requirements for methionine using corn gluten meal. Effects on blood nonesterified fatty

acids (NEFA) and beta-hydroxybutyrate (BHBA) were not significant. Zahra et al. (2006) reported that cows fed RPC had increased milk yield (+ 2.6 lbs/d) during early lactation, but effects of RPC supplementation on blood NEFA and BHBA along with liver composition were not significant.

Cooke et al. (2007) evaluated whether RPC supplementation could prevent and alleviate triglyceride accumulation in liver using a feed-restriction model in dry cows. Supplementation of RPC during feed restriction decreased plasma NEFA and decreased liver triglyceride accumulation, the latter by nearly 50% compared to controls. Furthermore, RPC supplementation during the refeeding period following feed restriction resulted in more rapid clearance of triglycerides from liver. Zom et al. (2011) reported that supplementation with RPC did not affect blood metabolites, but decreased liver TG content during early lactation; further examination (Goselink et al., 2013) of the changes in gene expression in liver from this study suggested that RPC supplementation resulted in increased expression of genes related to processing of fatty acids and VLDL assembly. Elek et al. (2008; 2013) determined that cows fed RPC produced 5.5 lbs/d more fat-corrected milk (9.7 lbs/d more milk) during early lactation, and these effects were underpinned by decreased liver triglyceride and circulating BHBA concentrations for cows fed RPC.

Recently, Zenobi et al. (2018a) fed cows either 0 or 60 g/d of RPC (17.3 g/d of choline chloride) for the last 17 d before expected calving through 21 d postpartum to cows fed either high-energy or controlled energy dry period diets. Cows fed RPC tended to have increased yields of milk (+ 4.8 lbs/d) and energy-corrected milk (+ 4.8 lbs/d) during the first 15 wk postpartum and tended to produce 4.6 lbs/d more milk over the first 40 wk of lactation. Concentrations of plasma NEFA and BHBA were not affected by RPC and liver TG content was similar between cows fed control vs. supplemented diets. Interestingly, cows fed RPC produced colostrum with greater IgG content and calves from cows fed RPC had greater weight gain from calving through 50 wk of age. Effects of RPC on production and metabolism were largely independent of prepartum nutritional strategy in this experiment. In a companion experiment, they sought to evaluate dose-response effects to RPC supplementation to levels up to 2X that fed in their transition cows study in a restricted-fed dry cow model; RPC supplementation linearly decreased liver TG accumulation during feed restriction (Zenobi et al., 2018b)

In addition to these results, there are other studies that have demonstrated either statistically significant (+ 5.3 lbs/d of milk, Scheer et al., 2002; + 6.4 lbs/d of milk, Pinotti et al., 2003; + 4.0 lbs/d of fat-corrected milk in one experiment and + 1.8 lbs/d of milk in a second experiment, Lima et al., 2007) or statistically nonsignificant (+ 5.1 lbs of fat-corrected milk, Janovick-Guretzky et al., 2006; + 1.8 lb/d of 3.5% fat-corrected milk, Leiva et al., 2015) production responses to RPC supplementation.

Methionine Supplementation During the Transition Period

Similar to the research conducted focused on choline supplementation to diets fed to transition cows, there is also a substantial body of work accumulated over the

past 20 years or so with regard to methionine supplementation during the transition period and early lactation. Overton et al. (1996) fed cows either 0 or 20 g/d of rumen-protected methionine (RPM) beginning 7 to 10 d before calving and continuing into lactation. Cows fed RPM produced 6.0 lbs more fat-corrected milk during early lactation. Socha et al. (2005) fed cows either 10.5 g/d of RPM or 10.2 g/d of RPM plus 16.0 g/d of rumen-protected lysine (RPL) beginning 14 d before expected calving and continuing through early lactation. Cows fed RPM plus RPL produced more milk during early lactation than those fed RPM alone; milk yield of cows fed the basal diets was intermediate. Supplementation of RPM and RPM plus RPL increased milk protein content when cows were fed 18.5% CP diets during the postpartum period; amino acid supplementation did not affect milk protein content when cows were fed 16% CP diets postpartum. Effects of amino acid supplementation on liver and energy metabolism were not evaluated in either the Overton et al. (1996) or Socha et al. (2005) studies.

Piepenbrink et al. 2003 determined the effects of feeding an analog of methionine (2-hydroxy-4-(methylthio)-butanoic acid; HMB) to periparturient cows on production and metabolism. They reported that feeding an intermediate level of HMB increased milk yield by 6.6 lbs per day; however, comprehensive evaluation of the effects of HMB on metabolism (circulating concentrations of NEFA and BHBA, liver concentrations of triglyceride and glycogen, in vitro assessment of liver propionate and palmitate metabolism) suggested that the production responses were not underpinned by changes in liver metabolism.

These responses were supported by those of Bertics and Grummer (1999), who used a research model similar to that described above for choline to evaluate responses of liver triglyceride accumulation during feed restriction and depletion during refeeding to HMB supplementation. In this study, HMB supplementation did not affect either triglyceride accumulation or depletion during the two phases of the experiment.

Ordway et al. (2009) evaluated feeding either the isopropyl ester of HMB (HMBi) or RPM to cows beginning during the prepartum period and continuing into early lactation. They determined that supplementation of HMBi and RPM did not affect yields of milk or fat-corrected milk (average milk yields were 95.7, 95.9, and 92.6 lbs/d for cows fed the basal diet, HMBi, and RPM, respectively); however, milk protein percentage was increased by feeding both HMBi and RPM. Effects of methionine supplementation on liver and energy metabolism were not determined in their experiment.

Preynat et al. (2009, 2010) fed cows RPM with or without intramuscular injections of folic acid and vitamin B12 during the transition period and early lactation. Feeding RPM did not affect milk yield (83.3 vs. 83.0 lbs/d for control vs. RPM, respectively), but increased milk crude protein percentage (2.94 vs. 3.04%). Interestingly, liver concentrations of triglycerides were actually increased in cows fed RPM in this study.

Osorio et al. (2013) fed cows either a basal ration or the basal ration supplemented with either HMBi or RPM beginning 21 d before expected calving and

continuing through the postpartum period. Cows fed methionine had large increases in milk yield compared to controls (+5.3 lbs/d for HMBi and +9.5 lbs/d for RPM); however, effects of the two sources of methionine on blood NEFA and BHBA and liver triglyceride content were not significant. Interestingly, cows fed supplemental methionine had greater phagocytosis in blood neutrophils harvested at 21 d postpartum, suggesting improved immune function. Further analysis of samples collected from this study suggested that cows supplemented with Met had better oxidative status as evidenced by lower plasma ceruloplasmin and serum amyloid A concentrations, greater plasma oxygen radical absorbance capacity, and greater liver concentrations of glutathione and carnitine (Osorio et al., 2014). Furthermore, these researchers detected alterations of gene networks in liver consistent with changes in oxidative status and inflammatory responses described above (Osorio et al., 2016).

Recently, Batistel et al. (2017b) fed cows RPM from 28 d before expected calving through 60 d postpartum Cows fed RPM had greater prepartum DMI (+ 2.6 lbs/d) along with greater postpartum (1 to 30 d) DMI (+ 3.7 lbs/d), milk yield (+ 9.1 lbs/d) and energy-corrrected milk yield (+9.7 lbs/d). Concentrations of plasma nonesterified fatty acids during the postpartum period were decreased in cows fed RPM. They also determined that calf birth weight was increased for cows fed RPM and that RPM upregulated AA transport and modulated the mTOR signaling pathway in the placentome (Batistel et al., 2017a)

Differential Responses to Choline and Methionine in Transition Cows

Results above suggest that both choline and methionine supplementation can improve performance of dairy cattle during the transition period, but that each may have distinct roles relative to metabolism. To determine the effects of choline and methionine more specifically on bovine hepatocytes, Chandler and White (2017) prepared primary monolayer hepatocyte cultures from neonatal calves and incubated the cells with increasing concentrations of either choline chloride or DL-methionine. They determined that increasing choline concentrations in the media increased secretion of VLDL into media and decreased the accumulation of reactive oxygen species in media. Furthermore, choline and methionine had differential effects on several of the enzymes related to one-carbon metabolism. Interestingly, there were no interactions detected between choline and methionine additions within this in vitro system.

Sun et al. (2016) fed cows either a control diet or diets supplemented with RPM, RPC, or both in a 2 x 2 factorial arrangement of treatments from 21 d before expected calving through 21 d postpartum. Both RPM and RPC increased prepartum and postpartum DMI and yield of fat-corrected milk during the postpartum period. Both RPM and RPC decreased postpartum concentrations of NEFA and BHBA and increased plasma concentrations of glucose. Furthermore, both RPM and RPC had similar effects on various indices of oxidative status. Interaction terms were not significant for virtually all outcomes, suggesting additivity of responses to RPC and RPM.

Summary

Choline and methionine have essential roles in various aspects of mammalian metabolism and are connected biochemically as part of one-carbon metabolism. Research conducted during the past 20 years supports roles for both choline and methionine in transition cow nutrition. Both choline and methionine generally increase productive performance during early lactation; however, they appear to affect transition cow metabolism differently. Choline appears to function primarily to increase triglyceride export from liver as very low density lipoproteins (VLDL) thereby decreasing liver triglyceride accumulation and improving liver energy metabolism. Methionine appears to alter oxidative status and immune function, thereby also affecting liver metabolism through inflammatory and immune mechanisms. The literature does not support an effect of methionine supplementation on liver triglyceride export. Recent results also suggest that both methionine and choline may have effects of relevance to the calf; methionine via modulation of placental function and choline via increased colostral immunoglobulin G, although more research is needed to fully understand potential epigenetic effects of these nutrients in the transition cow.

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