

PRODUCTIVITY IMPACTS OF STRATEGIES TO ENHANCE RUMINANT FIBER
DIGESTION

By

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ABSTRACT

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Strategies to improve forage fiber digestibility can increase dairy sustainability. Reducing lignin content through natural breeding or genetic engineering can improve DMI, neutral detergent fiber (NDF) digestibility and feed efficiency. A 60 mid-lactating cow experiment was carried out to compare production and nutrient digestibility responses to low-lignin vs. conventional alfalfa hay. The 3 treatments provided 0% (conventional), 50% (blend) and 100% low-lignin alfalfa; all diets contained 31.8% alfalfa on a dry matter (DM) basis. Low-lignin alfalfa decreased total-tract NDF digestibility and milk fat concentration in a dose-dependent manner. All other nutrient and production parameters were unaffected. Therefore, observed NDF digestibility and milk fat results suggest that the low-lignin alfalfa provided less physically effective fiber, failing to demonstrate obvious benefits. Another strategy for improving NDF digestibility involves use of different trace mineral supplements. In a small meta-analysis that included 8 studies and 12 comparisons in both sectors, beef and dairy, effects of hydroxy vs. sulfate trace minerals (TM) on nutrient digestibility and DM intake were investigated. Study design, sector and method of digestibility analysis were the factors included in the analysis. Hydroxy TM increased DM digestibility only in beef cattle and NDF digestibility, which was affected by digestibility analysis method. Total collection studies showed the greatest benefit, followed by use of undigested NDF as a digestibility marker, but 24-h *in situ* approaches did not show any significant response. In conclusion, adopting multiple strategies for optimizing fiber digestibility might help the dairy industry achieve greater feed efficiency goals.

This thesis is dedicated to my daughter, Zahra Ibraheem,
wife, Khola, and my parents for their unconditional support

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Chapter 1 – Literature Review

Effects of dietary characteristics on fiber digestibility in dairy cattle

Historically, ruminants have primarily relied on digesting forages to meet nutritional needs. They do this mainly with the help of ruminal microbes that act on the diet consumed, of which fiber is a major component. In terms of dairy rations, fiber can be described as the major component of forage cell walls. Nutritionally, it can be described as the fraction of feed that is relatively slowly digested or indigestible, taking up significant volume in the cow's gastrointestinal tract (Mertens, 1997). An enabling environment for ruminal microbes ensures adequate fermentation of dietary fractions and availability of absorbable nutrients for ruminants.

Both chemical and physical characteristics of a ration can affect the extent of nutrient utilization by dairy cows (Mertens, 1997). Fiber has multiple vital roles such as supplying nutrients, stimulating the rumen for rumination and fermentation, and forming ingesta bulk, thereby affecting passage rate and digestive health in general. Fiber is also responsible for stimulating significant chewing activity in cattle, leading to salivation which has an important buffering role in the rumen, where acidic products from dietary fermentation are formed. Roughage is essential for ruminants to achieve optimal production while maintaining a conducive environment for the microbes in the rumen (Allen, 1997). Since fiber inclusion levels, type and digestibility can alter dry matter intake (DMI), it is one of the most important practical considerations while formulating diets for lactating cows, especially highly productive cows. Dietary fiber is essential for maintaining rumen health; however, greater fiber inclusion in diet can lead to increased rumen fill and hence lesser DMI (Eastridge, 2006). Moreover, with relatively smaller increases in DMI as milk yield (MY) per cow increases, the importance of fiber digestibility and feed efficiency will continue to grow in future (Eastridge, 2006).

Inclusion of dietary fiber that is digestible enough to meet nutritional requirements of high-producing dairy cattle, provides adequate roughage for rumination, and is economical can be challenging. Therefore, dietary crude fiber, neutral detergent fiber (NDF), forage NDF (fNDF), undigested NDF in 30 hours (uNDF₃₀), 30-h in vitro neutral detergent fiber digestibility (30- h IVNDFD), physically effective NDF (peNDF) and physical effectiveness factor (pef) are some dietary fiber analytical parameters which are usually considered while formulating dairy rations. In vitro NDFD involves measuring fiber digestibility outside the cows, in a flask system containing a solution including ruminal fluid allowing for microbial digestion of a sample (Goeser et al., 2009). Several characteristics of a diet (e.g., forage particle size, forage-to-concentrate ratio, forage type, and dietary NDF) determine the amount of dietary physically effective fiber (Mertens, 1997). However, growing availability of a myriad of industrial by-products that are seldom classified as forages, having significant NDF content, may provide varying physically effective NDF. When including significant quantities of non-forage fiber sources (NFFS) or finely chopped forages in dairy rations, simpler descriptions like forage-to-concentrate ratio or dietary NDF concentration might be insufficient to quantify physically effective fiber in a diet. Hence, peNDF is one of the measures that aims to combine diet fiber chemical content and particle size to better assess dietary fiber adequacy in dairy cattle (Zebeli et al., 2012), and has gained growing attention recently.

Dietary physically effective fiber can affect nutrient and fiber digestibility by stimulating chewing activity and salivary buffer secretion, rumen motility and mixing, and maintaining appropriate functioning of the ruminal ecosystem (Allen, 1997; Zebeli et al., 2012). More than one technique of calculating the peNDF fraction of a diet exists. One method is to determine the product of NDF concentration and pef, which varies from 0 to 1 depending on the ability of

NDF from a given feedstuff to stimulate chewing (Grant, 1997; Mertens, 1997). Other methods include multiplying NDF content of a diet with DM found on top of a 1.18-mm screen (Zebeli et al., 2006). Calculation of peNDF by these various techniques might not yield the same values, however the values may still be useful for diet comparisons.

Dietary fiber adequacy, NDF, and peNDF of the diet are dependent on major fiber constituents in forages, namely cellulose, hemicellulose and lignin, which primarily form the plant cell wall. Cellulose and hemicellulose are considerably more digestible by ruminal microbes when compared to lignin, which is largely indigestible. Lignin is the fraction that gives rigidity to forages to help plants stay upright. Furthermore, apart from being mostly indigestible itself, cell wall lignin also reduces the digestibility of the other fiber fractions by reducing access of rumen bacteria to potentially digestible cell wall fractions (Jung et al., 2012). Thus, lignin can be an important determinant of fiber and nutrient digestibility. Increasing dietary peNDF (at least in a diet deficient in effective fiber) can improve rumen function and fiber digestibility by slowing down passage rate so there is ample time for microbial fermentation and NDF digestion in the rumen. The passage rate of fractions through the rumen, especially in high producing cows, can significantly influence ruminal NDF digestion (Firkins, 1997). Also, greater effective fiber in deficient diets may lead to more buffering by saliva which maintains ruminal pH, making it more conducive for cellulolytic bacteria; however, the precise relation between pH and growth of cellulolytic bacteria remains unclear at this stage.

Particle size of forages in the diet can also affect dietary peNDF, and in turn fiber digestibility. Physical characteristics of a diet are dependent on forage-to-concentrate ratio, type of forages and concentration used, ratio of non-forage fiber inclusion and particle size (Mertens, 1997). It has been reported that major causes of low dry matter intake and sub-acute ruminal

acidosis are extremely coarse and fine particle size in diets, respectively (Aschalew et al., 2019), thus highlighting the importance of balancing ration peNDF. Additional factors in combination to the ones mentioned above can influence dietary peNDF and by extension fiber digestibility.

Fiber sources commonly used in cattle diets can be broadly divided into forages and NFFS. Traditionally, fiber from forages has been heavily relied upon, although depending on availability, region and price, inclusion of NFFS varies in dairy rations. Shortages in forage sources due to environmental factors and dairies sourcing all their feeds rather than owning crop land are some of the reasons leading to greater use of NFFS in partially replacing forage fiber in rations (Armentano and Pereira, 1997; Firkins, 1997). Many NFFS included in dairy rations are plant by-products from processed human food (Armentano and Pereira, 1997) and other industrial processes like biofuel production. Inclusion of NFFS in dairy diets affords greater flexibility in achieving dietary fiber adequacy given yearly or seasonal changes in weather conditions and market trends. Non-forage fiber sources can successfully replace part of the forage and/or concentrate in a dairy ration depending on level of milk production (Bradford and Mullins, 2012)..

There is a wide variety of forages that can be fed to cattle. The most common forages for intensive dairy cattle production systems traditionally have included ensiled whole plant corn, alfalfa hay or haylage, and cool-season grasses. The proportions of different forages used in dairy rations are a function of a myriad of factors related to regional geography, land availability, and cost-benefit calculations. Covering an exhaustive list of forages used in dairies in different regions of the US or around the globe is beyond the scope of this thesis.

Numerous factors affect nutritive value of forages. For any given forage, hybrid or variety, maturity at harvest, processing, storage method, and duration of storage are some of the

major factors that can affect the dietary nutritive value and total-tract NDF digestibility (ttNDFD). Inclusion levels of various forage sources can be changed as well, depending on requirements; for example, alfalfa or grass hay inclusion can be modified depending on peNDF targets, fNDF requirements, and to regulate passage rate. This in turn can affect fiber and nutrient digestibility.

In addition to NDF, starch is another important nutrient vital for meeting energy requirement of lactating cows. Dietary inclusion of starch in rations varies depending on factors like region, ingredient availability, cost, production levels, and cow health concerns. Improving starch digestibility can lead to improved lactation performance and lower feed costs when grain prices are high (Ferraretto and Shaver, 2012). Also, it is not uncommon for one nutrient in a ration to affect digestibility of another nutrient in the rumen or beyond. The significance of this effect might be dependent on inclusion level of a given nutrient in a diet. Furthermore, the effect might be indirect; for example, starch concentration (de Souza et al., 2018) can affect cellulolytic bacteria by altering ruminal environment, which may affect NDFD. Dietary starch may lead to reduced cellulolytic microbe populations, although decreased NDFD may not be solely due to reduced pH, as substrate competition among facultative microbes using cellulose and starch can also be an important factor affecting NDFD (P.J. Van Soest, 1994). The importance of total mixed ration (TMR) starch levels for fiber digestion can be appreciated by the fact that despite significantly increased requirements for energy in early-stage lactating cattle, increasing TMR energy levels by using more starchy grain is not recommended (NRC, 2001). Also, dairy cows consuming significant quantities of rapidly fermentable grains usually have lower ruminal pH due to increased production of acid in the rumen and have lesser dry matter

intake (Allen, 2000). Hence dietary starch can potentially affect fiber digestibility depending on inclusion levels.

Fats, and more specifically fatty acids (FA), are an important component of dairy rations despite comprising less than 6% of diet dry matter in lactating cow rations. Dietary fats usually refer to ingredients or compounds consisting mainly of FA (both long and short chained), found in the form of triglycerides, non-esterified FA, and phospholipids. In many dairy rations, most of the fat content may be attributed to addition of oilseeds, commercial fat supplements such as animal and animal-vegetable blends, dry granular products, and rumen-protected fats (NRC, 2001). Supplemental fats are typically used to increase the energy density of a ration to support milk production (Jerred et al., 1990) and milk fat content (Palmquist and Jenkins, 1980).

However, fats or lipids have been documented to adversely affect rumen fermentation (Jenkins, 1993), indicated by factors such as depressed DMI, fiber digestion and milk fat concentration (NRC, 2001). Historically it has been reported that fat inclusion in dairy rations can reduce fiber digestibility, however properties of fat such as saturation and esterification maybe an important factors mediating impacts on fiber digestion (Palmquist and Jenkins, 1980). Several aspects of dietary fat can potentially alter fiber digestion in the rumen, ranging from fat inclusion levels, fat type, basal diets, and FA proportions in a supplement. These will be addressed later in the chapter.

As for all organisms, minerals are essential for cattle to support normal functions such as growth, reproduction, and structural development. Minerals are categorized as either macrominerals such as calcium, phosphorous, sodium and chloride, or microminerals such as zinc, copper, manganese and iron, depending on whether they are required in gram or milligram quantities per day, respectively (NRC, 2001). The safety margin between adequate and toxic

dietary concentrations of microminerals and trace minerals (TM) is much narrower than most other nutrients. In the past decades, different chemical forms of TM have been used to supplement cattle, including inorganic oxides (which were more common in the past) and sulphates, hydroxides, and amino complexes (organic). It is entirely possible that the TM source used in a diet may affect digestion of other nutrients like fiber, in addition to potential impacts on production parameters. For example, a number of studies (Faulkner and Weiss, 2017; Daniel et al., 2020) reported greater in vivo NDFD in lactating dairy cows supplemented with hydroxy TM when compared to sulfate TM. Others (Guimaraes et al., 2021) have observed similar effects in beef steers fed hydroxy TM whereas (Genther and Hansen, 2015) did not observe significant effects on in situ NDFD. Variation in ruminal solubility (Caldera et al., 2019) among different TM sources seems to be an important factor that might be responsible for the observed results. Therefore, choice of TM source may be a factor in determining nutrient digestibility in both dairy and beef cattle.

Effects of type of fiber source

Neutral detergent fiber from forages and NFFS accounts for most of the NDF in a dairy cow ration. Most crop processing involves fractionating different product streams, and the fiber-rich component is often undesirable for monogastric applications but suitable for ruminants (Bradford and Mullins, 2012). Many NFFS also contain a substantial amount of protein in addition to NDF. Additionally, NFFS are frequently low in lignin making its fiber highly digestible and therefore a useful energy source (Boddugari et al., 2001). Despite high NDF concentration, the effectiveness of fiber and fiber particle size from NFFS generally is not comparable to NDF from forage, thus requiring careful consideration especially when formulating at higher inclusion levels. It must be noted, however, that considerable between-

batch variation in chemical and physical characteristics can result in significant ttNDFD variation for these by-products (Firkins, 1997). Common NFFS included in dairy rations include soy hulls, beet pulp, citrus pulp, brans, cottonseeds and DDGS. Cottonseeds and DDGS also have a significant NDF concentration ~ 50% and ~44% respectively. As indicated by Clark and Armentano (1993), the traditional guidelines that dairy cattle diets should contain at least 27% NDF with 75% of this NDF derived from forages may not hold well when the definition of forages and concentrates are unclear. For example, categorizing by-product feeds like linted cottonseed, DDGS, beet pulp and soy hulls - which can be high in both fiber and energy - as either forages or concentrates is complicated (Clark and Armentano, 1993). Therefore, describing dairy ration in terms of forage-to-concentrate may not be useful, especially in case of higher NFFS inclusion in diets. Fibrous NFFS may have an NDF value similar to forages but can rapidly pass through the rumen like concentrates (Bradford and Mullins, 2012). Therefore, peNDF, which considers both physical and chemical characteristics of fiber, can help nutritionists in terms of feeding adequate fiber in diets for optimal production in high milking cows. Based on 3 important studies (Allen, 1997; Firkins, 1997; Mertens, 1997) the NRC (2001) concluded that NDF from NFFS was, on average, half as physically effective as NDF from forages. This shows that NFFS has at least some effective value and can be used to replace forages to an extent. Elaborating further, the NRC (2001) recommends increasing total dietary NDF by 2% units for every 1% unit decrease in dietary fNDF under 19%.

Holt et al. (2010) reported little effect of including NFFS like soy hulls and beet pulp with either conventional or brown mid rib (BMR) varieties of corn silage on in vivo NDFD, DMI, MY and 3.5% FCM. This is despite significant differences for in vitro NDF degradability between the 2 silage types (42.2 vs 31.2%) included in the diets, which had similar total NDF

content. The factors that can help explain observed results include relatively high inclusion (23.6% of DM) of finely chopped high-quality alfalfa (CP = 21.3%; NDF = 38.3%) in relation to silages (25% of DM) fed in these diets. Also, the relatively low inclusion rates (soy hulls = 4.2%; beet pulp = 4.2% of DM) of NFFS compared to the forages is another factor that can help explain the limited variation in NDFD, DMI and production parameters. More importantly, the greater passage rate due to high inclusion of finely chopped, good quality alfalfa hay may help to explain similar NDFD results whether NFFS were included or not. In case of finely chopped forages, NRC (2001) recommends increasing NDF concentration of diets. This indicates the importance of ingredient profile and dietary characteristics like peNDF and fNDF in determining both utility of NFFS and fiber digestibility in diets. Depending on the passage rate, ruminal NDFD of NFFS can vary significantly (Bhatti and Firkins, 1995), with lesser digestibility as a result of greater passage rates. A review by Grant (1997) seems to agree with this, reporting an 8% increase in passage rate when soybean hulls inclusion in a pelleted mix for dairy cows was increased from 50 to 95%. Altered ttNDFD of diets after including NFFS may be explained by a greater proportion of potentially degradable NDF and smaller particle size of most NFFS, which can lead to higher quantities of potentially available NDF to pass from rumen before it can be digested (Firkins, 1997). Conversely, it is also speculated that improvement in NDFD for 5 studies after addition of coarse hay may have been due to increased retention time of NFFS (Grant, 1997). Hence, when replacing considerable amounts of forages with NFFS, interactions between source, characteristics and amount of dietary forages with NFFS can affect ruminal and total tract NDFD, passage rate and ultimately milk production in dairy cows.

Choice of forage sources

Decisions regarding the type and inclusion rate of forages on a dairy farm depends, to a large extent, on availability, price, nutrient profile, consistency, ease of storage and the animal group in question. The possibility of long-term storage for ensiled whole plant corn with minimal spoilage makes it a practical choice of forage for lactating cows, especially for high producing cows. Corn silage is the main contributor of fiber and, to lesser extent, energy for most dairy cattle in North America. Similarly, the storage of hay in the form of compact dry bales or haylage harvested at optimum maturity allows for a consistent supply of an ingredient with high NDF digestibility and crude protein. Thus, inclusion of alfalfa hay or haylage in lactating cow rations and alfalfa hay in calf rations is common. Also, dry hay bales allow for efficient transportation on a DM basis, making it a viable option for many dairies procuring ingredients.

Physical and chemical characteristics of dietary ingredients and their interactions can have a large effect on dry matter intake (DMI) of lactating cows (Allen, 2000). Typically, dairy cows undergo negative energy balance in early lactation, which can affect peak MY. Hence, maximizing energy intake during early lactation is dependent on maximum DMI, which in turn may depend on physical fill or the volume limits of the rumen. Dairy cows require an adequate amount of dietary NDF for optimal ruminal function and milk yield (Oba and Allen, 1999a). Replacing one source of NDF with another more digestible one for improved production efficiency while maintaining ruminal function is often a successful strategy for supporting increased milk production.

Emergence of forage varieties like BMR corn with improved ruminal NDFD along with NFFS reinforce the importance of balancing for NDFD and physical effectiveness of fiber. The BMR mutations in corn have led to reduced lignin concentrations and significant improvement

of in vitro NDFD with little change in NDF concentration (Cherney et al., 1991). In a study by Oba and Allen (2000), high producing cows were fed low (29%) and high (38%) dietary NDF concentrations using either BMR (bm3) or its isogenic control, which differed substantially in NDFD measured in vitro (55.9 vs. 46.5% at 30 h). Intake was significantly increased by both low dietary NDF ($P < 0.001$) and BMR silage ($P = 0.02$) when compared with high dietary NDF and control corn silage, respectively. Similarly, milk yield was significantly increased by low dietary NDF ($P < 0.01$) and BMR silage ($P < 0.01$) compared with higher dietary NDF and control corn silage, respectively. Finally, FCM (3.5%) did not differ significantly among the two dietary NDF levels ($P = 0.51$) but BMR silage tended ($P = 0.06$) to increase FCM (3.5% fat) regardless of dietary NDF treatment (low NDF = 35.6 vs. 34.3; high NDF = 35.8 vs. 32.6 kg/d). The results show that the greater NDFD of BMR corn silage may be responsible for decreased ruminal fill and greater passage rate, leading to increased DMI regardless of dietary NDF concentration. Greater DMI and milk yield for BMR silage regardless of dietary NDF levels, with little change in milk fat yield, indicate that greater forage NDFD can have productivity benefits. Interestingly, greater milk solid-not-fat concentration (SNF%) for BMR silage were observed for the high NDF diet only, indicating that incorporating forages with greater NDFD in diets with relatively high NDF content might result in more optimized milk component production.

In the case of alfalfa, increasing maturity is associated with increased cell wall material (CWM), leading to decreased crude protein (CP) concentration and overall digestibility (Albrecht et al., 1987). Apart from increased stem:leaf ratios, the concentration of CWM increases dramatically in stems (60%) with maturity compared to leaves (10%) (Albrecht et al., 1987). Increases in CWM and lignin can help explain overall reduced digestibility of alfalfa with progressing maturity. Similar to BMR corn, different methods have been undertaken to produce

varieties of alfalfa that have lesser lignin concentrations compared to conventional varieties. High-quality alfalfa varieties like Hi-Gest (Alforex) with comparatively lesser lignin content have been developed via conventional plant breeding, through selection to increase alfalfa digestibility or maintain forage quality over a wider harvest window. A reduction in lignin concentration in these varieties is achieved by increasing leaf to stem ratio through natural breeding. On the other hand, genetically engineered (GE) reduced-lignin alfalfa varieties have been developed like HarvXtra (U.S. Dairy Forage Research Center/ARS-USDA, Forage Genetics International, Pioneer HiBred and The Samuel Roberts Noble Foundation, Inc.) by deactivating a lignin synthesis pathway enzyme called caffeoyl CoA 3-O-methyltransferase (USDA-APHIS, 2014; Staudenmeyer et al, 2017). Both high-quality (or low lignin) and reduced-lignin alfalfa varieties may potentially increase fiber digestibility in dairy cows; however, feeding trials using dairy cows are very limited. On the other hand, a few beef animal studies utilizing reduced-lignin alfalfa hay have shown ambivalent results. Staudenmeyer et al. (2017) showed no treatment differences in BW change, ADG and DMI when comparing reduced-lignin variety (HarvXtra, HX-4114) vs conventional (variety WL336HQRR) when feeding beef heifers. Another preliminary beef steer study (Karls et al., 2017) fed reduced-lignin (HarvXtra), high-quality (HiGest 360), or conventional (LegenDairy XHD) alfalfa baleage ad libitum, resulting in small numerical ADG differences which were not statistically significant. However, ttNDFD tended ($P = 0.08$) to increase from conventional (33.9%) and high-quality (35.0%) to reduced-lignin (40.3%) alfalfa varieties.

Forages with lesser digestibility seem to limit voluntary DMI (VDMI) by physical distention of the gastrointestinal tract, or more specifically the reticulorumen; however, this inhibition of VDMI appears to reduce with increasing digestibility of feeds (Allen, 1996). As

fiber ferments slowly in the rumen compared to non-fiber nutrient fractions, its retention time in the rumen is greater than other fractions, resulting in fiber exerting a greater effect on physical fill of the rumen (Oba and Allen, 2000). Thus, unsurprisingly, increasing the peNDF content of a corn silage-based diet can improve digestibility of fiber (Yang and Beauchemin, 2005) especially when cows are peNDF deficient. Organic matter (OM) fermentation produces acid at the rate of ~ 74,000 meq/d, however salivary buffer is secreted at approximately half that rate (~ 41,000 meq/d; (Allen, 1997). Sufficient dietary peNDF is an important factor, as discussed before, that affects chewing activity and salivary buffering in cows. When reduced nutrient digestibility is expected due to fast passage rate, inclusion of coarse grass hay can be considered. Conversely, finely chopped high quality alfalfa hay or NFFS can be included in diets that predominantly include conventional corn or sorghum silage with relatively poor in vitro NDFD. Thus, optimizing forage NDFD while considering minimum physical effectiveness of fiber or dietary peNDF can maximize milk production efficiency in terms of balancing passage rate, NDFD and DMI.

Fiber composition and ruminal microbes

Approximately 60-70% of the energy supplied by a dairy cow ration comes from carbohydrates, which are utilized by both ruminal microbes and the host, while also maintaining gastrointestinal health (NRC, 2001). Carbohydrates can be divided into nonstructural carbohydrates (NSC) and structural ones. Nonstructural carbohydrates include sugars and starch, while structural carbohydrates refer to fiber, predominantly plant cell walls commonly measured as NDF. Despite significant use of concentrates in ruminant rations, cell wall digestibility is an important factor regulating energy intake and energy value of forages (Jung and Allen, 1995). Cell wall matrices can be described as complex assembly of phenolics, proteins and

polysaccharides like cellulose, hemicellulose, lignin (Hatfield et al., 2017) and pectin. Cellulose and hemicellulose are largely digestible by ruminal microbes, however, lignin is virtually indigestible. The proportion of these fractions in forages varies with forage type, varieties, and maturity, affecting digestibility of the cell wall. Lignin content usually increases with forage maturity, resulting in reduced digestibility of cell wall carbohydrates (Demeyer, 1981) by physically protecting structural carbohydrates against microbial attack (Allen and Mertens, 1988). Therefore, lignin is a key factor to focus on for improving forage digestibility since it directly impacts ttNDFD.

According to Jung and Allen (1995), ferulic acid bridges serving as cross-linkages between lignin and cell wall polysaccharides (cellulose and hemicellulose) may be an important step before lignin exerts negative effects on ttNDFD. Plants having low ferulate cross-linking showed greater in vitro rumen NDFD, plus the negative effects of cross-linking were twice than that of lignin concentration among a number of perennial grasses (Casler and Jung, 2006). This introduces an interesting point, whereby NDFD of forages may not be solely determined by lignin content but also its cross-linking with other fiber components (cellulose and hemicellulose).

Four basic steps for forage cell wall digestion include reduction of particle size, ruminal microbial enzymatic hydrolysis of the cell wall, fermentation of polysaccharides to yield volatile fatty acids (VFA), and fermentation end-product absorption (P.J. Van Soest, 1994). The first 2 steps are related to mastication and fiber composition of a diet, respectively. Microbes provide VFA and protein to ruminants by attacking, degrading and then fermenting cell wall NSC in forages (Varga and Kolver, 1997). Some major cellulase-producing fibrolytic ruminal microbes include *Fibrobacter succinogenes*, *Ruminococcin flavefaciens* and *Ruminococcus albus* (Stewart

and Hobson, 1997) whereas *Butyrivibrio fibrisolvens* is considered to be more important in the hydrolysis of hemicellulose (Varga and Kolver, 1997). Fungi comprise only about 8% of ruminal microbial biomass (Orpin and Joblin, 1997) which may be attributed to slower generation time (Varga and Kolver, 1997). Fungi are capable of both cellulase and hemicellulase activities. Thirdly, data from in vitro experiments (Gijzen et al., 1988) suggest that protozoa contribute 19 – 28% of total cellulase activity. However, it seems fibrolytic bacterial probably account for the majority of fiber digestion in the rumen.

Microbes tend to attack damaged areas of forages, which occur during chopping, processing, and finally mastication. Physical availability or exposure of substrates to ruminal microbes seems to be an important factor that determines cellulose digestibility (Weimer, 1993). Weimer (1993) also points out that it appears microbes only attack plant cells from the inside, or through the lumina. This seems to be in line with Jung and Allen (1995), who propose that deposition of lignin moves from the primary to secondary wall following polysaccharide deposition. This may help explain the occurrence of microbial degradation of plant cell walls from the inside (lumen) out with the lignified primary cell wall or middle lamella escaping degradation (Engels, 1989). Overall, it seems reasonable that lignin concentration reduction with the aim of improving digestibility without losing agronomic performance is a strategy to be considered. Regarding forage crop improvement, Jung and Allen (1995) suggested reducing cell wall concentration for grasses based on considerably greater NDF content of grasses, whereas for legumes, increased cell wall digestibility might be a better target for improvement.

Role of starch in fiber digestibility

Carbohydrates can be divided into structural and NSC, and starch accounts for 50-100% of NSC in most feedstuffs. Dietary starch is one of the key energy sources for dairy cattle in

most intensive production systems. Starch degradation rate and extent of digestion in the rumen can be affected by source, processing methods, and animal characteristics like DMI and passage rate. Ruminal degradability varies among sources; for example, barley is more degradable than millet and corn has a greater degradability than sorghum (Herrera-Saldana and Huber, 1989). Around 50% of the energy value of corn silage is attributed to starch based on calculations from NRC (2001) according to Ferraretto and Shaver (2012), making starch an important determinant of energy supplied to the animal. Grain processing methods like cracking or grinding to reduce particle size during harvest can increase ruminal starch digestibility and fermentation (NRC, 2001). Thus, it can be reasonably expected that variation in ruminal starch digestibility can potentially affect other nutrients in the rumen and beyond.

A comparison of soy hull and corn grain treatments fed at 30% of the diet showed increased DMI, milk yield, milk protein, milk fat, and energy corrected milk for the corn grain treatment (Boerman et al., 2015). In this study, lower dietary starch seemed to affect high producing cows more than low producing cows, which were able to maintain milk production when fed the low dietary starch. Interestingly, the soy hull diet, with a greater total NDF concentration, resulted in increased apparent ttNDFD (43.9 vs 27.2%). Moreover, a significant interaction between treatment and preliminary milk yield was observed for NDFD, which may be explained by greater DMI and passage rate for high producing cows, leading to reduced retention time. Other factors that can help explain treatment NDFD differences in this study include greater ttNDFD of soy hulls compared to forage NDF, which formed the majority of the NDF in the corn grain diet. Lastly, high-starch diets can influence microbial populations or reduce ruminal pH to cause reduced ttNDFD. Decreases in DMI, NDFD, and microbial yield due to low ruminal pH can lead to decreased milk production (Allen, 1997). In batch cultures, ruminal

cellulolytic bacteria like *F. succinogenes* grown on cellobiose showed little growth at pH < 6.0; similarly, *R. albus* showed significantly reduced growth at pH < 6.0 (Russell and Wilson, 1996). It seems that strategies of resisting pH change, either by constant maintenance of intracellular pH as in *R. succinogenes*, or by allowing decreases of intracellular pH as in *R. albus*, result in inhibition of cellular metabolism (Russell and Wilson, 1996). The latter strategy indicates that ruminal microbes might have enzymes that do not tolerate acidic conditions well. Also, it is possible that high concentrations of VFA may contribute to inhibition of bacterial growth and metabolism.

Overall, the effects of high starch diets on ruminal pH and microbes ultimately influence NDFD. For high producing cows, however, strategies for increasing starch digestibility (like grinding) might exert lesser effects on the ruminal environment due to greater DMI and passage rate. This might help explain why high producing cows may be able to tolerate high levels of starch in diets without clinical signs or adverse effects.

Fatty acid impacts on fiber digestion

With increasing genetic potential of dairy cows to produce milk, higher energy levels in diets have been a requirement to maintain optimal milk production in early and mid-lactation, not to mention the recovery of body stores in mid to late lactation. Fat inclusion in diets has been one way to increase energy density (Pantoja et al., 1994). Dietary fat inclusion levels and relative concentrations of saturated and unsaturated fatty acids making up the fat content can affect nutrient digestibility despite relatively lower inclusion in diets compared to other nutrients. Maximizing fatty acid (FA) content of diets can lead to inhibition of cellulolytic bacterial activity, leading to reduced fiber digestibility. Also, different types of fatty acids (saturated and

unsaturated), depending on relative and absolute concentrations, can potentially affect ruminal fiber digestion.

Even though a majority of unsaturated FA will be biohydrogenated in the rumen before passing into the intestine, unsaturated FA seem to inhibit cellulolytic microorganism metabolism more than saturated FA, potentially affecting ruminal fiber digestion (Eastridge and Firkins, 1991). It is indicated in the NRC (2001) that unsaturated FA tend to have a greater negative impact on rumen fermentation when compared to saturated FA. A meta-analysis by Weld and Armentano (2017) using 38 published papers evaluated effects of dietary fat addition on ttNDFD in lactating cows in response to 108 fat-supplemented treatments. The meta-analysis concluded that while dietary SFA 12-14 carbons in length decreased ttNDFD, inclusion of calcium salts of long chain fatty acids (LCFA) may have resulted in increased ttNDFD. Moreover, no other 16- or 18-carbon fat source seemed to affect ttNDFD significantly, nor were effects on ruminal NDFD apparent. Comparing a relatively pure palmitic acid (PA) supplement (99% C16:0) with a control (soy hulls) fed at 2% of the diet, Piantoni et al. (2013) showed that PA supplementation increased milk yield, milk fat yield and feed efficiency. Moreover, increased ttNDFD was observed for PA supplementation (39.0 vs 35.7%; $P < 0.001$). Improved fiber digestibility could be a result of greater plasma cholecystokinin concentration (CCK; 19.7 vs 17.6 pmol/ L; $P < 0.001$); CCK inhibits gastrointestinal motility, potentially leading to greater ruminal NDF retention time and increased ttNDFD (Piantoni et al., 2013). Therefore, effects of fats in general on NDFD are variable at best, and dependent on a host of factors including inclusion rate, FA type, fat supplement type, DMI, passage rate and possibly lactation performance.

Source of micromineral supplementation

Microminerals or trace minerals are vital for proper functioning of bodily processes like metabolism, fertility, production, and immunity (S.K. Kvidera, 2019). However, as the term implies, trace minerals like Cu, Zn and Mn are required in minute quantities and are mostly supplemented in excess of requirements. Trace minerals can be toxic, or at least counter-productive, if given in great excess. In vivo studies using Zn-deficient diets for animals showed that Zn was adequate for rumen microorganisms, and excess dietary Zn concentrations may decrease DM digestibility (Somers and Underwood, 1969; Arelovich et al., 2000). Source of micromineral or trace mineral supplementation can affect nutrient digestibility despite the trace concentrations involved. Different mineral sources that have been or are being used in dairy rations include sulfates (inorganic), oxides, hydroxides, amino acid complexes (organic) or a combination of these sources. Chemical characteristics of mineral sources like ligand types can affect mineral solubility among different gut compartments, leading to varying availability of minerals for absorption (Daniel et al., 2020) and possible interactions with gut microflora.

Since different trace mineral sources may have different solubility in water, this can affect reactivity with potential antagonists and ruminal microorganisms as well (Caldera et al., 2019) and most of the antagonistic reactions would likely take place in the rumen (S.K. Kvidera, 2019). Excess Zn, for example, may bind to bacterial surfaces, inhibiting bacterial attachment to cellulose and thereby reducing cellulose hydrolysis (Eryavuz and Dehority, 2009). Trace minerals like Cu, Mn and Zn negatively affect cellulose digestion in vitro even in small concentrations, although results from in vivo studies have been less consistent (Genther and Hansen, 2015). This would mean that a high availability or solubility of minerals like Cu and Zn from a given source (e.g., sulfate salts) can alter microbial fermentation in dairy cattle when

compared to other sources of trace minerals like hydroxides or amino acid complexes (Caldera et al., 2019).

Cow production levels and disease

Apart from external factors related to nutrition or environment, it is possible that cow factors such as production level can also affect nutrient digestibility. When comparing the effects of 30% dry ground corn with 30% soyhulls, Boerman et al., (2015) found treatment \times preliminary milk production for MY, milk fat, milk protein and 3.5% fat-corrected milk. More importantly, higher producing cows responded with a greater increase in ECM on ground corn than cows with lesser yields, indicating an effect of cow production level. Conversely, high producing cows' response to reduced starch was greater than the response shown by low-producing cows. It can be postulated that low-producing cows were able to maintain milk production on soy hulls due to slower passage rate, which allowed for greater NDFD. In cases of diseased or recovering animals, more digestible diets might help in faster passage rate and greater increase in DMI, especially during the convalescence period. If energy requirements are low then high dietary NDF may not limit DMI, and conversely, low dietary NDF can limit DMI in case energy requirement are not fulfilled (Oba and Allen, 2000). In an another study by Bradford and Allen (2004) comparing starch fermentability between dry corn and HMC, it was shown that greater fermentability of starch leads to milk fat depression in low producing cows, however high producing cows were spared. Again, this cow production response can partially be explained by greater passage rates in high producers allowing for lowered fermentation of starch. Furthermore, higher rate of ingesta flow from the rumen may also prevent a buildup of acidic products beyond a certain level. Animal effects, especially related to milk production do seem to

affect nutrient digestibility, which may vary depending on nutrient sources and processing methods.

SUMMARY

Fiber from forages constitutes a large percentage of a dairy cow's diet, providing energy and allowing for optimal rumen function. However, growing forages for feeding a lactating cow requires availability of land, financial investment and a suitable environment which makes it an operation of its own. With decreasing land resources and environmental concerns around crop farming, there is a need to use forages efficiently, while also maximizing utilization of by-products and co-products from food and other industrial processes. Strategies for improving fiber digestibility in cattle includes improving forage genetics and making use of mutation, as in the case of BMR corn silage, and adapting ration formulations by wisely combining ingredients available. Improvements in biotechnology promise increasing availability of digestible forages like reduced-lignin alfalfa hay or BMR sorghum. A deeper understanding of ruminal function and potential impacts of mineral and fat supplements can aid in optimizing fiber efficiency, ensuring economical and sustainable dairy production systems.

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Chapter 2 – Impacts of low-lignin alfalfa on intake, digestibility, and productivity of lactating Holstein cows

ABSTRACT

Increasing nutrient digestibility in dairy cattle is generally expected to improve farm economics, environmental sustainability, and milk production efficiency. Since forages form the bulk (40 – 60%) of a dairy cow's diet, there are potentially large benefits to be realized from increasing the digestibility of forages. Recent efforts have focused on improving fiber digestibility of alfalfa, one of the major forage crops, by both natural breeding and genetic engineering aimed at reducing lignin content. However, we are not aware of any research to date using low-lignin (**LL**) alfalfa in a lactating cow ration. In this study, 60 mid-lactating cows (119 \pm 19 days in milk, DIM) with parity ranging from 1-4 were used in randomized complete block design. Cows were housed in tie stalls and the study was carried out with 2 cohorts with 30 cows each. A common diet was fed during a 2-wk covariate period, followed by a 6-week treatment period. Treatment diets were formulated to be identical except for 32% (DM basis) of the diet, comprised of a) conventional alfalfa hay (**CON**), b) a 50:50 blend of conventional and LL alfalfa hay (**BLD**), or c) LL alfalfa hay (**LL**). Feed intake and milk yield were recorded daily, while milk composition and body weight were determined on 1 d/wk. Fecal, orts and ingredient samples were collected on the last 3 days of the treatment period to determine nutrient digestibility using undigested NDF as an internal marker. There was no significant effect of treatment on DMI, or DM, CP, or starch digestibility. Significant treatment effects were observed for NDF digestibility (59.0, 55.9 and 53.5 \pm 1.84% for CON, BLD and LL, respectively). Milk yield and body weight were not significantly affected by treatments, but milk fat concentration was significantly decreased by LL alfalfa (3.57, 3.49 and 3.40 \pm 0.04% for

CON, BLD and LL, respectively). The NDF digestibility and milk fat concentration responses were unexpected but suggest that LL alfalfa may have disrupted ruminal function in the diets evaluated here. Marked differences in peNDF calculations (Mertens, 1997) were not observed between treatment diets (24.5, 23.9 and 23.0% for CON, BLD and LL, respectively), although a trend was evident. Overall, the results indicate that the safety margin of effective fiber should be considered strongly when using forages with increased digestibility to prevent possible alteration in ruminal function.

INTRODUCTION

Alfalfa is primarily grown for dairy cattle and horses. In terms of acreage and production value, alfalfa is the 4th largest crop in the U.S. (Fernandez-Cornejo et al., 2016). Compared to other forages, alfalfa is high in protein (20-22%) and low in fiber content, although alfalfa fiber is relatively more lignified than fiber in cool-season grasses or corn. Lignin itself is considered to be indigestible. Moreover, its binding with other cell wall polysaccharides like cellulose and hemicellulose reduces digestibility of these potentially digestible compounds, thus reducing NDF digestibility (NDFD; Moore and Jung, 2001).

Increasing the fiber digestibility of alfalfa while retaining desirable plant attributes like high yield, resistance to lodging, pests, diseases and herbicides, could provide opportunities to improve feed efficiency and milk potential per hectare. Conventional breeding techniques have been used in the past (Alforex, Woodland, CA) to develop alfalfa varieties with greater leaf:stem ratios, which also indirectly decreases lignin concentration. It is well established that lignin content of forages increases with maturity; however, yield improves with maturity as well. This presents a dilemma for farmers trying to maximize yield while maintaining optimal forage digestibility to promote milk production. In addition to natural breeding, more recently advances in biotechnology have accelerated development of genetically engineered (GE) glyphosate-tolerant and reduced-lignin varieties of alfalfa like HarvXtra (U.S. Dairy Forage Research Center/ARS-USDA, Forage Genetics International, Pioneer HiBred and The Samuel Roberts Noble Foundation, Inc). These reduced-lignin varieties were developed by deactivating a lignin synthesis pathway enzyme, resulting in a ~15% reduction in lignin content which might not be possible via natural breeding (Barros et al., 2019). Reduced-lignin varieties show acceptable in vitro NDFD values at most advanced stages of maturity compared to conventional varieties, and

therefore harvest can be delayed, potentially improving yield without compromising on fiber digestibility. This wider window for harvesting both high-quality and reduced-lignin varieties is especially attractive for managing constraints such as limited equipment availability or rain, which can make it difficult to harvest alfalfa at the optimal time in many parts of the country.

Genetic selection has enabled modern dairy cows to produce considerably more milk than they did 60 years ago. Nutritionists must develop feeding strategies that enable the cow to express her genetic potential. In high-producing cows, physical fill of the gut is an important limitation to DMI (Allen, 1996) which is primarily caused by forage NDF (fNDF). Increasing ttNDFD, especially for forages, can reduce the filling effect of fNDF, leading to increased DMI and potentially greater milk yield. This effect has been most clearly demonstrated by the BMR mutation in corn (Oba and Allen, 2000). On average, BMR corn silage had 0.8 % unit less lignin and 9% units greater 30-h in vitro NDFD (45.3 % vs. 36.8%) than isogenic conventional corn silage (Oba and Allen, 1999b); BMR silage significantly increased DMI (25.6 vs. 23.5 kg/d), milk yield (41.7 vs. 38.9 k/d), and 3.5% fat-corrected milk yield (41.0 vs. 38.4 kg/d). This is in line with results from Oba and Allen (2000), which showed significant increases in DMI (24.7 vs. 23.9 kg/d at low dietary NDF; 22.9 vs. 21.5 kg/d at high dietary NDF) for cows consuming BMR corn silage vs. conventional silage. Similarly, milk yield was significantly greater for cows fed BMR (36.9 vs. 33.5 kg/d at low dietary NDF and 35.8 vs. 32.6 kg/d at high dietary NDF). Hence, it is possible that lower-lignin alfalfa would improve DMI and milk yield in a similar manner.

Meeting minimum requirements for physically effective fiber (without greatly exceeding them) allows for maximal DMI and can theoretically enable greater milk yield in high-producing cows. On the other hand, increasing digestibility of fNDF reduces its physical effectiveness,

increasing risk for problems such as milk fat depression and subclinical acidosis. Thus, proper balancing of dietary NDF and fNDF is vital for optimal productivity and health of dairy cattle. Although there are some studies involving steers (Staudenmeyer et al., 2017; Reiter et al., 2020) and sheep (Mertens, 2009), we are not aware of any published trials using reduced-lignin alfalfa varieties in diets for lactating dairy cows. The objectives of this study were to determine whether feeding lactating cows lower-lignin vs. conventional alfalfa at about 1/3 of the diet would alter DMI, nutrient digestibility, or production of milk and milk components.

MATERIAL AND METHODS

Two fields (University of Nebraska research farm, Mead, NE) were seeded at 16.8 kg of pure live seed per hectare with 3 different alfalfa varieties on September 5, 2019. In one field, 5.9 hectares of the control variety (**CON**; Dairyland Hybriforce 3400) was planted. The second field was designated for the low-lignin (**LL**) varieties and divided in half with 3.2 hectares planted to 54HVX42 HarvXtra and the other 3.2 hectares planted to AFX 460. Prior to planting, weeds were killed using Roundup PowerMax (glyphosate) on August 29, 2019 at 1.33 kg of glyphosate acid per hectare. All 3 plots were harvested at a target of 10% bloom throughout the 2020 growing season. The newly established plots generated very poor yields for cutting 1, so hay harvested from cuttings 2 (6th July), 3 (4th August), and 4 (21st September, 2020) were used in the feeding study. Each bale harvested was sampled using a drill probe hay sampler (Best Harvest Inc., Largo, FL) along with a standard power drill to collect 10 core samples per bale (Table 2.1) from different locations. After grinding to pass through a 1-mm screen (Wiley Mill; Arthur A Thomas Co., Philadelphia, PA) samples were analyzed for nutrient profile by near infrared spectroscopy (NIRS) by Cumberland Valley Analytical Services Inc. (Waynesboro,

PA). Samples were analyzed for DM, CP, ADICP, NDICP, ADF, NDF, lignin, starch, crude fat, ash, Ca, P, Mg, and K. Relative feed value was also calculated.

After hay transport to Michigan State University, conventional hay was ground in a sequence designed to maximize consistency of nutrient profiles over time by alternating bales from different cuttings. Likewise, LL hay was ground to maximize nutrient consistency, but also to evenly blend the two varieties used as sources of LL hay. To evaluate the general impact of lower lignin content and greater NDFD, two varieties of LL hay with different approaches to development were blended. Ground hay was crudely mixed after grinding and further blending occurred during feed-out.

Cows and treatments

Experimental procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (protocol # 202000102). Sixty mid-lactating cows (119 ± 19 DIM) with parity ranging from 1 – 4 were used in a randomized complete block design (RCBD). Cows were housed in tie stalls with continuous access to individual automated water dispensers. The study was carried out with 2 cohorts of 30 cows each. The experiment consisted of a 2-week covariate period and a 6-week treatment period. During the covariate period, all cows were fed a common diet containing conventional hay from a different source than that used during the treatment period. At the end of the covariate period, cows were blocked by milk yield, DIM, milk fat % and milk protein % from the 4-day collection period. Treatments ($n = 3$) were then randomly assigned to cows within each block ($n = 20$).

The total mixed ration (TMR) was formulated in accordance with NRC requirements and fed once daily (0800 h). Treatment diets were formulated to be identical in composition except for the different types of hay, each fed at 31.8% of DM. Treatments were 1) conventional alfalfa

hay (CON); 2) a 50-50 blend of conventional and low-lignin alfalfa hay (BLD), and 3) low-lignin alfalfa (LL).

Data collection and sampling procedure

As-fed intake of cows was measured daily throughout the covariate and treatment periods by calculating the difference between the weight of feed offered and refusals during a 24-h period. Depending on refusals weight, feed offered was adjusted daily to provide 110% of expected intake for each cow. Total mixed ration and ingredient samples were collected once during the second week of the covariate period and once per week during the treatment period. In the last week of the treatment period, in addition to the weekly TMR sample, ingredient samples were collected for the last 3 days of the period. All TMR and hay samples were collected in duplicates and one replicate was used for particle size analysis using the Penn State Particle Separator (PSPS) while the remaining replicate was stored for analysis. Ingredient samples collected during the covariate period, or the last 3 d of the treatment period were composited within period before analysis by Dairy One Forage Laboratory (Ithaca, NY). These samples were analyzed for DM, CP, ADF, aNDFom, starch, ash and 240-h undigested aNDFom (uNDF₂₄₀). Fecal samples were collected from every cow every 9 h in the last 4 d of the treatment period until 8 samples were collected, representing every 3 h of a 24-h day. Fecal samples were composited within a cow before analysis. Similarly, 12.5% of the orts were collected daily and these samples were composited across 3 d within a cow. Composited fecal and orts samples were sent to Dairy One Forage Laboratory (Ithaca, NY) for analysis of DM, CP, ADF, aNDFom, starch, ash, and uNDF₂₄₀ as described above.

Body weights were recorded once per week (1600 h) during the covariate and treatment periods. Milk samples were collected for all 3 milkings during the final 4 d of the covariate

period and 1 d per wk during treatment period, and analyzed for fat, protein, lactose, milk urea nitrogen (MUN), and somatic cell count (SCC) at CentralStar Cooperative (Grand Ledge, MI).

Statistical analysis

Prior to statistical analysis, DMI, milk component and yield data for each cow were averaged by week. Analysis of composited samples of orts and feces over the 3-day collection phase at the end of the treatment period resulted in one data point per cow. Statistical analysis was performed using the GLIMMIX procedure in SAS (version 9.4) in models that included fixed effects of parity group (lactation 1 vs. 2+), treatment, cohort and their interaction and random effects of block nested within cohort. For variables with repeated measures over time, the model also included the fixed effects of covariate value, covariate \times treatment, cohort, cohort \times treatment, week, treatment \times week, parity \times week, and treatment \times parity \times week. Parity, covariate, and interactions were removed from the model when effects were $P > 0.15$, except that treatment \times week was retained in all repeated-measures models, and first-order factors were retained in a model when an interaction of that factor was retained. Data points with a Studentized residual exceeding an absolute value of 4 were excluded as outliers. Significance and tendencies were declared at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

RESULTS

The study was designed to feed alfalfa at a relatively high proportion of the diet (8.7 kg/d per cow; Table 2.3) in order to increase the likelihood of observing treatment effects. On average, conventional hay had $\sim 1.7\%$ units greater aNDFom concentrations than low-lignin bales (45.0 vs. $43.3 \pm 0.64\%$). The average lignin content difference between conventional and low-lignin bales was 0.8% units (7.9 vs. $7.1\% \pm 0.23$; Table 2.2). Little difference in CP content

for the two types of hays was observed (mean 19.0%). On the whole, the macronutrient profiles for conventional and low-lignin hay were reasonably similar.

Dry matter intake and nutrient digestibility

A treatment effect was observed for DMI ($P < 0.01$; Table 2.4) where DMI increased for LL compared to CON. No interactions with parity group were observed (interaction $P = 0.56$). However, covariate DMI from the pre-treatment period did interact with treatment (Figure 2.1); as covariate DMI increased, LL had a more positive effect on DMI. Digestibility of DM was not affected by treatment ($P = 0.11$), however an interaction between treatment and cohort was significant ($P < 0.001$) and a tendency for an interaction was observed for parity and treatment ($P = 0.054$; Table 2.4). Treatment significantly affected ttNDFD ($P < 0.01$), with CON increasing ttNDFD compared to BLD ($P = 0.04$) and LL ($P < 0.001$). BLD and LL treatments did not differ ($P = 0.17$) in ttNDFD. Furthermore, cohort and the interaction of treatment and cohort also had significant effects on ttNDFD ($P < 0.01$), where cohort 2 showed greater overall ttNDFD (58.7 vs $54.8 \pm 0.01\%$). Starch digestibility was not affected by treatment ($P = 0.46$) or parity \times treatment ($P = 0.87$). Finally, CP digestibility was also unaffected by treatment ($P = 0.16$), although significant parity \times treatment ($P = 0.015$) and cohort \times treatment interactions were observed ($P < 0.01$). However, slicing by parity did not reveal significant treatment effects on CP digestibility for either parity ($P \geq 0.20$).

Milk production and body weight

There was no significant effect of treatment on milk yield ($P = 0.71$; Table 2.5). A significant effect of treatment ($P < 0.01$) was observed for milk fat %. Differences were significant between CON and LL ($P < 0.001$) with CON cows showing increased milk fat content (3.57 vs. $3.40 \pm 0.04\%$). The BLD treatment was intermediate (3.49% fat) and tended to

differ from both CON ($P = 0.09$) and LL ($P = 0.07$). Fat yield was not affected by treatment overall ($P = 0.56$), but there was a tendency for week \times treatment interaction ($P = 0.062$) with only week 4 exhibiting significantly different milk fat yield ($P = 0.02$). Least-square means for milk fat yield for CON, BLD and LL in week 4 were 1.45, 1.31, and 1.42 ± 0.04 kg/d, respectively. An interaction of covariate fat yield and treatment was observed as well ($P < 0.01$). The interaction plot between treatment and covariate fat yield (Figure 2.5) shows BLD increasing fat yield proportionally with covariate fat yield compared to the other treatments, which exhibited a lesser increase, similar to the covariate ECM \times treatment plot.

No treatment effects were observed on milk protein concentration ($P = 0.54$), however interaction with cohort was significant ($P = 0.01$). Treatment did not significantly impact milk protein yield ($P = 0.16$). A week \times treatment interaction ($P < 0.01$) was found, with week 4 again being the only week in which treatments differed ($P < 0.001$). Milk lactose concentration was not significantly affected by treatment ($P = 0.62$), and lactose yield was also unaffected by treatment ($P = 0.71$). Overall treatment effects were not significant for energy-corrected milk (ECM) yield ($P = 0.55$), but a significant covariate ECM \times treatment interaction was observed ($P = 0.02$, Figure 2.2). BLD showed a proportional increase in ECM yield with covariate ECM yield, whereas CON and LL exhibited a lesser increase.

Treatment had no overall effect on MUN ($P = 0.86$), however there was tendency for week \times treatment interaction for MUN ($P = 0.06$).

Somatic cell score was unaffected by treatment overall ($P = 0.97$), but there was a significant week \times treatment interaction ($P < 0.01$). Week 1 showed a tendency ($P = 0.08$) and week 4 showed a significant effect of treatment ($P < 0.01$; Figure 2.3). The LL treatment had significantly greater SCS in week 4 compared to the other two treatments.

No effect of treatment on body weight change was observed ($P = 0.35$), however a significant week \times treatment interaction for body weight change ($P = 0.03$) was found. When sliced by week, treatments only affected BW change in week 1 ($P < 0.01$; Figure. 2.4). LSM for CON, BLD and LL for week 1 were -22.7, -8.4 and -8.6 ± 3.50 kg/wk.

DISCUSSION

Increased DMI by cows on LL compared to CON diets is perhaps not surprising given that greater inherent digestibility of fiber can lead to increased DMI, although impacts on milk yield are less consistent. While investigating effects of NDF content and alfalfa quality on milk production, Beauchemin (1991) reported increased DMI for cows fed early bloom vs mid bloom alfalfa in dairy rations. However, despite different DMI for the two hay qualities, stage of alfalfa maturity had no effect on milk production for diets formulated at similar NDF concentration. Also, the difference between average NDF content of early and mid-bloom alfalfa in the study mentioned was greater (38.8 vs 47.6%) than in the alfalfa used for our study (43.3 vs 45.0%). However, when diets increased from 31 to 37% NDF, milk yield decreased linearly due to decrease in net energy intake, presumably due to increased fNDF in diets. A comparison of 3 stages of alfalfa hay, early vegetative, late bud and full bloom, by Llamas-Lamas and Combs (1990), with varying NDF (36.1, 51.7 and 51.7%) and lignin content (4.9, 7.1 and 8.2%) revealed increased DMI for early vegetative vs the other two maturities. Alfalfa hay was not fed at similar proportions of the diet in order to maintain a similar alfalfa NDF intake, hence forage-to-concentrate ratio varied across diets for (early vegetative = 68:21; late bud = 53:47; full bloom = 45:55). This variation in forage-to-concentrate ratio might become an important factor in determining DMI at higher concentrate ratios due to energy density (Mertens, 1987). More

importantly, greater DMI and NDFD in case of early vegetative maturity did not translate to significantly increased milk yield although there was a tendency for increased fat yield (Llamas-Lamas and Combs, 1990). This illustrates that even when an alfalfa hay-based diet with greater DMI, NDFD and significantly lower lignin %, does not guarantee greater milk yield.

Comparing bermudagrass with alfalfa at two inclusion levels (15 vs 30% DM) including a corn silage control, West et al. (1997) reported that alfalfa increased DMI, milk yield, and 3.5% FCM yield ($P = 0.001$). Moreover, DMI, milk yield, and 3.5% FCM yield were also greater for the low alfalfa hay inclusion diet vs high alfalfa inclusion diet ($P = 0.01$). DM and CP digestibility remained unchanged among all comparisons, however in contrast to milk responses, apparent ttNDFD tended to be least for low alfalfa hay diet (37.7%) when compared to high alfalfa diet (40.8%) or bermudagrass diets. Perhaps, greater DMI for the low alfalfa diet due to reduced retention time compensated for reduced apparent ttNDFD. This is in line with the reduced ttNDFD for LL diets, which showed greater DMI and similar production parameters as other treatments. Also, despite greater ttNDFD of bermudagrass, its NDF content is significantly greater than alfalfa (80.7 vs 48.1%), with lesser amounts of the most digestible nutrient fractions (non-fiber carbohydrate and protein).

It is worth mentioning a beef heifer feeding study (Staudenmeyer et al, 2017) comparing reduced-lignin HarvXtra (HX-4114) vs conventional alfalfa (WL336HQRR). Similar to our findings, no treatment differences were observed for BW change, ADG or DMI. Although CP content of the alfalfas were similar (RL = 20.51; conventional = 20.70%) compared to our study, NDF (RL = 30.61; Conventional 31.17%) and lignin (RL = 5.69; Conventional = 6.02%) content was noticeably different (Table 2.2). Additionally, a preliminary beef steer baleage feeding study (Karls et al., 2017) comparing reduced-lignin (HarvXtra) vs low-lignin (HiGest 360) vs

conventional (LegenDairy) alfalfa did not detect any variety effects for ADG or feed efficiency. These findings are similar to ours. In their review on development of reduced-lignin alfalfa, Barros et al. (2019) reported similar NDF content to Staudenmeyer et al (2017) for HarvXtra (30.5%) and conventional alfalfa (31.3%). However the lignin content of HarvXtra (3.0%) and conventional alfalfa (4.0%) differed from both Staudenmeyer et al. (2017) and our study. On the other hand, Xu and Min (2022) reported similar NDF for conventional (Gunner; NDF = 46.8%) and low-lignin alfalfa (Hi-Gest 360; NDF = 45.4%) to this study. Overall, it seems that the NDF and lignin content of alfalfa used in this study was noticeably greater when compared to others. This is likely influenced by different cutting numbers and intervals across studies. Moreover, a comparison between bale core analysis (Table 2.1) and ground hay analysis (Table 2.2) also indicates an increase in NDF during grinding, which can be attributed to leaf loss.

Treatment effects for ECM yield were not significant; however, a significant covariate ECM \times treatment interaction was present. Figure 2.2 shows that responses for BLD seemed to increase proportionally with covariate ECM compared to the other treatments which exhibited a lesser increase. This is not easily explained, given that BLD was the intermediate treatment and was expected to result in responses between that of the other two treatments. Importantly, there was a parity \times treatment interaction ($P = 0.03$) which revealed a significant increase in ECM yield for multiparous cows only. Response of multiparous cows to CON, BLD and LL were 42.5, 40.5 and 43.6 ± 0.77 kg/d, respectively. Again, BLD stands out here when compared to the other treatments for reasons that are not obvious and interestingly LL shows the greatest ECM. No effect of fair vs. high quality alfalfa hay (NDF = 39.6 vs 33.6%; CP = 17.9 vs 21.9%) was reported for ECM yield by Eun et al. (2014).

In this study, milk fat concentration was the only milk component showing significant effects across treatments. Increasing inclusion of LL alfalfa decreased fat content in a dose-dependent manner (means of 3.57, 3.49, and 3.40% for CON, BLD, and LL, respectively). Effects of alfalfa hay quality or maturity on milk fat content seem to be variable among studies. Comparing responses to prime, grade-1, and grade-2 alfalfa hay (NDF = 28, 37.8, and 47.1%; CP = 18.5, 14.7, and 12%; lignin = 4.19, 6.03, and 7.47%, respectively), Van Zyl et al. (2014) did not find a significant difference in milk fat concentration or yield. Eun et al. (2014) reported increased milk fat content for fair quality alfalfa hay compared with high quality alfalfa hay (NDF = 39.6 vs 33.6%; CP = 17.9, vs 21.9%) when feeding high-moisture corn (HMC), but this difference was lost when feeding steam-flaked corn. This illustrates the possibility of a interaction between ingredients in a given diet and a variety of quality of alfalfa hay on milk production parameters. West et al., (1997) found increased milk fat content in response to high alfalfa hay diets compared to low hay diets (30% vs 15%) for Holstein cows, which might be explained by greater effective fiber supply in high hay diets. Effect of alfalfa hay inclusion on fat yield was not significant, however. Milk fat content was unchanged across diets composed of early vegetative, late bud, and full bloom alfalfa hay (Llamas-Lamas and Combs, 1990). Milk fat yield tended to be greater for early vegetative hay since cows were able to maintain milk fat concentration while significantly increasing milk yield. Beauchemin (1991) reported no change in milk fat content when comparing early and mid-bloom alfalfa hay, although dietary NDF had a significant linear increasing effect on milk fat content.

Previous findings on alfalfa hay quality and maturity impacting milk fat fail to completely explain the observed results in this study. The CON alfalfa provided slightly more aNDFom than the LL alfalfa (45.0 vs 43.3%), which might help explain improved milk fat

concentration for CON. This, along with the improved total-tract NDFD for CON, suggest an improved ruminal environment for this diet. In addition to small differences in aNDFom supply, the somewhat less digestible fiber chemistry in the CON alfalfa may have resulted in more physically effective fiber for that treatment, as well. We propose that cows fed CON likely had greater ruminal pH, providing a better environment for bacteria critical to fiber degradation (Russell and Wilson, 1996) and fatty acid biohydrogenation. However, ruminal pH and rumen dynamics were not assessed in this study.

For this study, ttNDFD was the only nutrient digestibility parameter that was significantly affected by treatment; surprisingly, it was the CON diet and not the LL diet that resulted in the greatest total-tract NDFD. A partial explanation may be that the lignin content between the conventional and lower lignin alfalfa was not different enough to impact digestibility. Nonetheless, the relationship between lignin content and fiber digestibility is not perfect, as the level of ferulate cross-linking between lignin and other plant cell components has a substantial impact as well. Casler and Jung (2006) reported that cross linking had twice the negative effects on fiber digestion as lignin concentration in several perennial grasses. Unfortunately, we do not have information on potential structural differences between the varieties used herein.

Oba and Allen (1999b) reported a 9.7-unit greater 30-h in vitro NDFD for BMR silage when compared to conventional corn silage (49.1 vs. 39.4%), however an increase of only 2.2 units (33.1 vs. 30.9%) was observed for total tract NDFD. In most BMR treatments, DMI was increased, equating to lesser retention time in the rumen, decreasing the potential for in vivo improvements in extent of fiber digestion. Given that DMI differed between CON and LL, we cannot rule out the possibility that the LL alfalfa failed to be retained in the rumen as well as the

CON alfalfa, which would have decreased residence time and extent of ruminal fiber digestibility. This is supported at least in part by 30-hr IVNDFD results of CON and LL hay (46.8 vs 48.8%). An alternative explanation is that BLD and especially LL diets did not have sufficient effective fiber, resulting in depressed rumen pH leading to lesser ttNDFD. We note that silage used for all the treatments was from BMR corn, and dietary aNDFom differed by 1.5 units between CON and LL (Table 2.3). This difference was primarily due to inherently different aNDFom levels of the hay used in the study.

CONCLUSION

Low-lignin alfalfa hay had no effect on milk yield, or milk components, with the exception of a dose-dependent decrease in milk fat concentration. However, LL increased DMI compared to CON. Low-lignin hay had no effect on total-tract DM, CP, or starch digestibility, but surprisingly, it decreased total tract NDF digestibility. These results can likely be attributed to insufficient effective fiber for LL treatments, perhaps due to primary effects on fiber kinetics (particle breakdown and passage) and secondary effects on rumen pH. Regardless of the mechanism, these results do not support preferential feeding of LL hay to lactating cows and suggest that diets based on LL alfalfa should perhaps be formulated with a greater safety margin for physically effective fiber content.

APPENDIX

Table 2.1. Nutritional profile of conventional and low-lignin alfalfa hay bale core samples. Values are means of 11 - 22 samples.

	Dairyland Hybriforce 3400	54HVX42 HarvXtra	AFX 460
Nutritional profile, % of DM except where noted			
DM (% as-fed)	90.6	92.2	91.1
CP	21.1	20.8	19.5
ADF	31.9	28.8	30.2
aNDFom	39.1	35.5	36.0
Lignin	6.7	6.2	6.7
Ether Extract	2.9	2.8	2.7

Table 2.2. Nutritional profile of conventional (CON) and low-lignin (LL) alfalfa hay after grinding.

	CON	LL
Nutritional profile, % of DM except where noted		
DM (% as-fed)	88.9	88.6
CP	18.9	19.0
ADF	35.4	34.1
aNDFom	45.0	43.3
Lignin	7.9	7.1
Starch	2.30	3.95
Fatty acids	1.74	1.93
30-h IVNDFD	46.8	48.8
30-h IV DM digestibility	75.9	77.6

Table 2.3. Ingredient and nutritional composition of treatment diets containing conventional (CON), low-lignin (LL), or a 50:50 blend (BLD) of alfalfa hay. Values are actual mean inclusion rates from feed management software.

	CON	BLD	LL
Ingredient, % of DM			
CON Alfalfa	29.3	14.9	-
LL Alfalfa	-	16.0	29.5
Corn silage (BMR)	20.3	20.4	21.2
High moisture corn	13.4	12.9	14.1
Corn grain	11.8	11.5	11.3
Protein, mineral, and vitamin supplement	8.7	8.4	8.3
Corn distillers grains	5.6	5.4	5.3
Soybean hulls (pelleted)	4.9	4.8	4.7
Soybean meal	3.3	3.2	3.1
Energizer 2 tallow	2.5	2.4	2.3
AjiPro L	0.3	0.3	0.3
Nutrient, % of DM (unless otherwise specified)			
DM (% as-fed)	56.7	56.8	56.3
CP	17.5	17.7	17.5
ADF	19.6	19.6	19.0
aNDFom	29.2	28.7	27.7
Starch	25.0	24.7	25.7
Fatty acids	4.3	4.3	4.3

Table 2.4. Intake and digestibility responses to diets with conventional (CON), low-lignin (LL), or a 50:50 blend (BLD) of alfalfa hay.

	Treatment			SEM	P-value
	CON	BLD	LL		Treatment
DMI, kg/d	25.0 ^a	25.4 ^{ab}	25.7 ^b	0.30	< 0.01
DM digestibility, %	71.3	69.5	69.3	1.63	0.11
NDF digestibility, %	59.0 ^a	55.9 ^{ab}	53.5 ^b	1.36	< 0.01
Starch digestibility, %	96.5	96.2	96.7	0.40	0.46
CP digestibility, %	70.4	68.6	68.4	2.02	0.16

Table 2.5. Milk production and composition responses to diets with conventional (CON), low-lignin (LL), or a 50:50 blend (BLD) of alfalfa hay.

	Treatment			SEM	P-value	
	CON	BLD	LL		Treatment	Trt × wk
Milk yield, kg/d	41.7	41.6	42.2	0.52	0.71	0.20
Fat %	3.57 ^a	3.49 ^{ab}	3.40 ^b	0.04	0.003	0.62
Fat yield, kg/d	1.43	1.42	1.41	0.03	0.56	0.06
Protein %	2.91	2.89	2.92	0.02	0.54	0.28
Protein yield, kg/d	1.21	1.19	1.23	0.02	0.16	< 0.01
Lactose %	4.88	4.88	4.89	0.01	0.62	0.58
Lactose yield, kg/d	2.04	2.03	2.06	0.03	0.71	0.19
ECM ¹ , kg/d	40.9	441.3	41.4	0.60	0.55	0.10
MUN, mg/dL	13.4	13.2	13.2	0.25	0.86	0.06
SCS	0.97	0.99	1.14	0.15	0.97	< 0.01
Body weight change, kg/wk	-0.12	1.49	1.53	0.94	0.35	0.03

¹Calculated using the formula: = (0.327*milk yield) + (12.95*fat yield) + (7.65*protein yield).

Figure 2.1. Interaction plot for covariate (pre-treatment) dry matter intake (CovDMI) and treatment for cows fed diets containing conventional (CON), low-lignin (LL) or a 50:50 blend (BLD) of alfalfa hay. Regressions are plotted from the 10th to the 90th percentile of the CovDMI distribution. Dotted lines represent the 95% confidence intervals of the regressions.

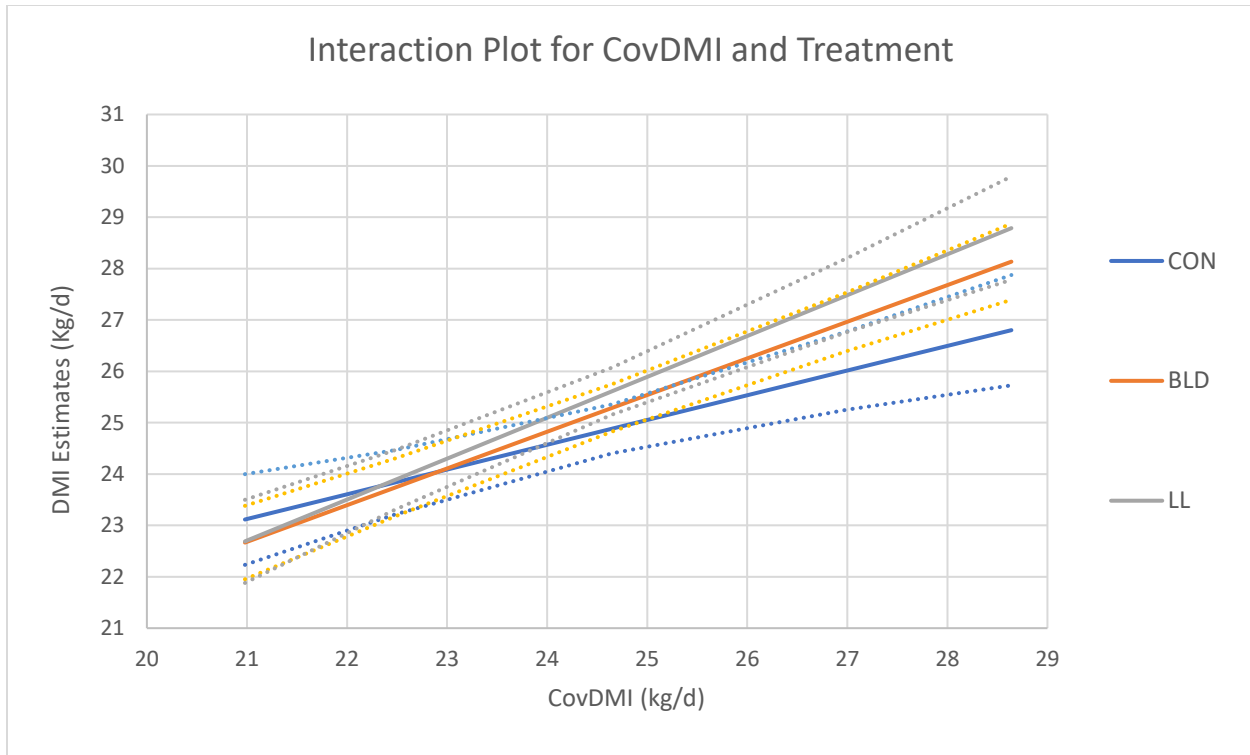


Figure 2.2. Interaction plot for covariate (pre-treatment) energy-corrected milk yield (CovECM) and treatment for cows fed diets containing conventional (CON), low-lignin (LL) or a 50:50 blend (BLD) of alfalfa hay. Regressions are plotted from the 10th to the 90th percentile of the CovECM distribution. Dotted lines represent the 95% confidence intervals of the regressions.

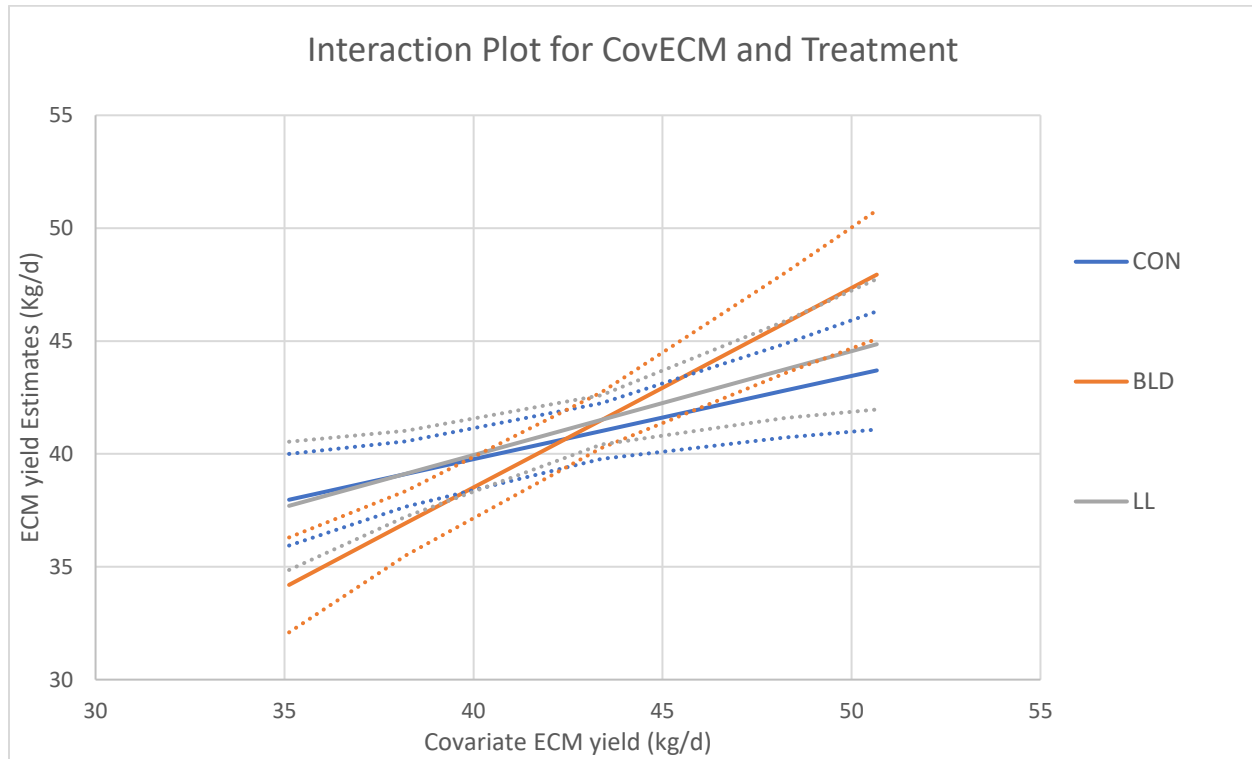


Figure 2.3. Week \times treatment interaction plot for Somatic Cell Score (SCS) for cows fed diets containing conventional (CON), low-lignin (LL) or a 50:50 blend (BLD) of alfalfa hay. Values are LS means \pm SEM.

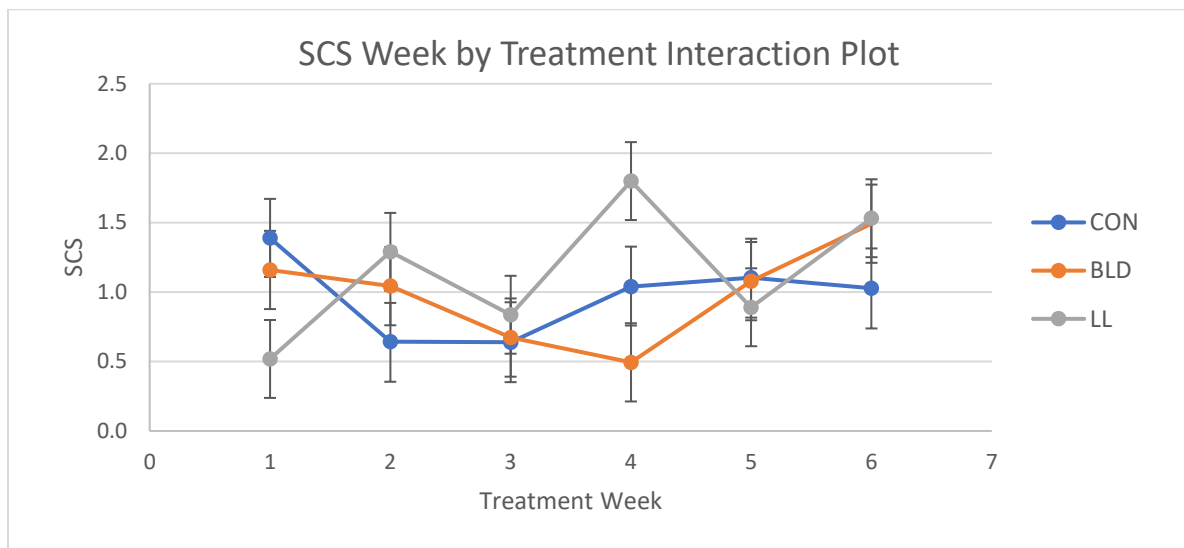


Figure 2.4. Week \times treatment interaction plot for body weight change for cows fed diets containing conventional (CON), low-lignin (LL) or a 50:50 blend (BLD) of alfalfa hay. Values are LS means \pm SEM.

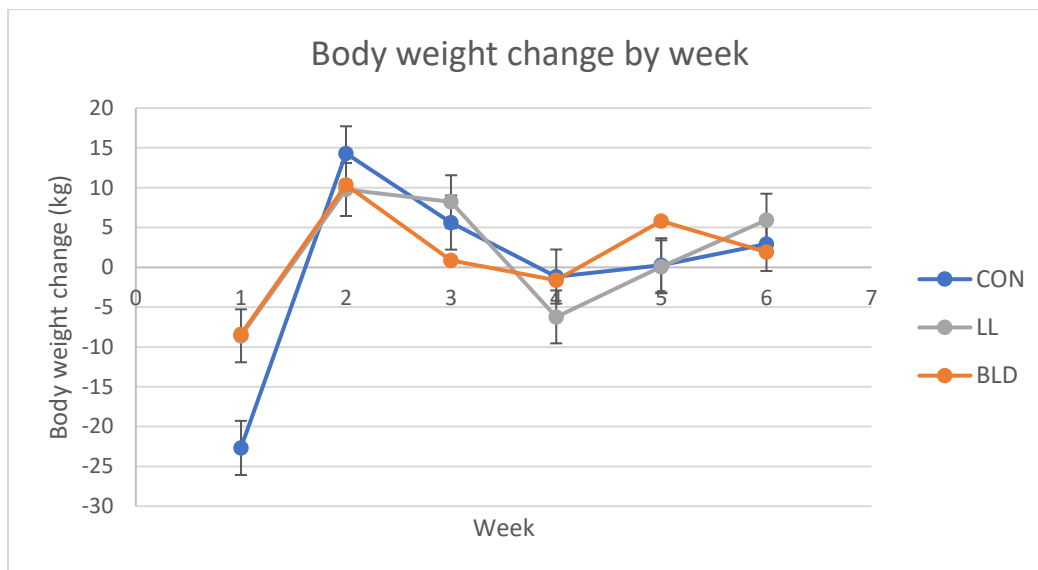
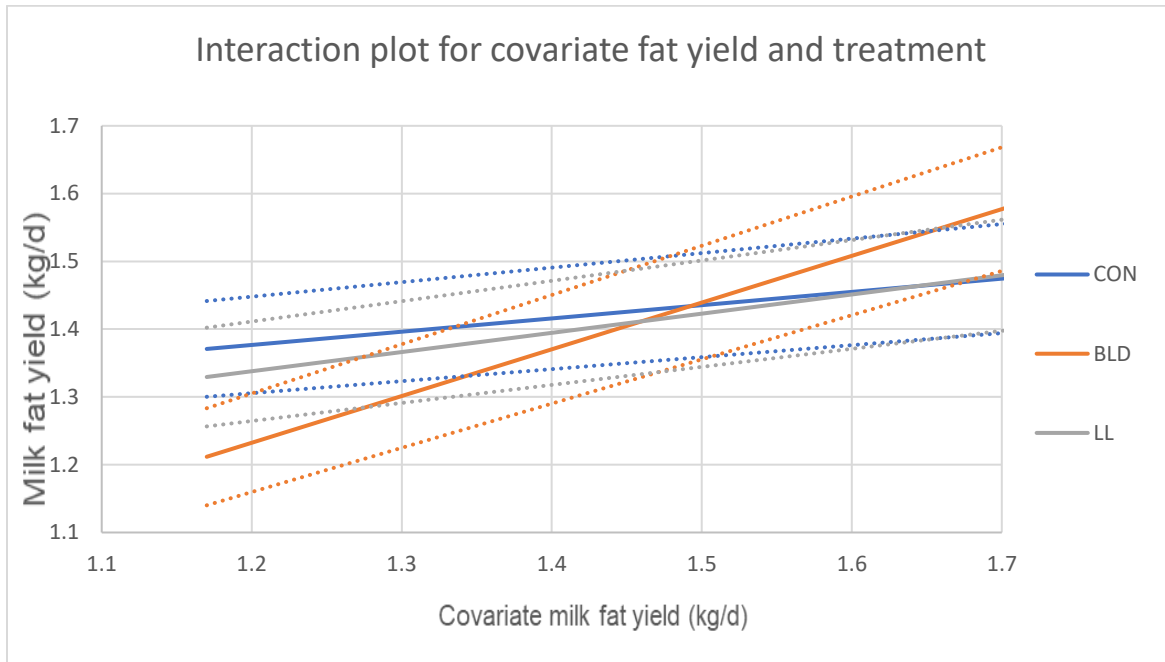


Figure 2.5. Interaction plot for covariate (pre-treatment) milk fat yield (Cov. Milk Fat Yield) and treatment for cows fed diets containing conventional (CON), low-lignin (LL) or a 50:50 blend (BLD) of alfalfa hay. Regressions are plotted from the 10th and 90th percentile of the covariate milk fat distribution. Dotted lines represent the 95% confidence intervals of the regressions.



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Chapter 3 – Effects of trace mineral source on nutrient digestibility: A meta-analysis

ABSTRACT

Trace mineral (TM) source can potentially alter nutrient digestibility through effects on microbial populations. A meta-analysis was conducted to determine whether sulfate vs. hydroxy (IntelliBond) sources of supplemental Cu, Zn and Mn had any effect on dry matter intake (DMI), dry matter (DM) digestibility and neutral detergent fiber (NDF) digestibility. All available cattle studies (8 studies, 12 comparisons) were used to assess responses (hydroxy mean - sulfate mean). Factors included in the analysis were method of digestibility analysis (total collection, marker-based, or 24-h in situ), study design (randomized design or Latin square), beef ($n = 5$) vs. dairy ($n = 7$) cattle, and days on treatment; these factors were retained when $P < 0.05$. DM digestibility was increased by hydroxy TM in beef ($+1.64 \pm 0.35$ units, $P < 0.001$) but not in dairy models ($+0.16 \pm 0.13$ units, $P = 0.29$). NDF digestibility increased significantly with hydroxy TM ($P = 0.02$), but digestibility assessment method influenced this response. Studies using total collection or undigested NDF as flow marker showed a significant increase ($+2.68 \pm 0.40$ units, $P < 0.01$, and $+1.08 \pm 0.31$ units, $P = 0.02$, respectively) in NDF digestibility; but studies utilizing 24-h in situ incubation did not detect any change (-0.03 ± 0.23 units, $P = 0.89$). These observations may reveal differences in precision of measurement or may indicate mineral impacts beyond the rumen; total collection is considered the gold standard method. Hydroxy TM did not affect DMI per animal ($+0.3$ kg/d; 0.35 ; $P = 0.43$) or per unit of body weight ($+0.04\%$; 0.048 ; $P = 0.47$). In conclusion, hydroxy TM do not appear to affect DMI but, depending on type of cattle and method of measurement, can increase DM digestibility and NDF digestibility, which may be

explained by differences in solubility of the TM sources in rumen differentially affecting fermentation.

INTRODUCTION

Minerals are inorganic elements, which like other nutrients are essential for physiological functions and need to be replenished mainly through the diet to avoid health issues. Major functional roles of minerals are to provide structure for the body, contribute to regulatory mechanisms, and catalyze various enzymatic reactions in the body (Suttle, 2010). Macrominerals are required in greater quantities than others, whereas TM are required in significantly lesser quantities. Typically only 0.01% of an organism's total mass is constituted by TM (Rabiee et al., 2010). Nevertheless, TM are required and essential for biochemical processes supporting growth, maintenance, milk production, fertility, and immunity (Hilal et al., 2016).

Adequate TM concentrations in feed are often taken for granted since TM effects on animal physiology are routinely underestimated (López-Alonso, 2012). This demonstrates the importance of considering trace mineral concentrations in diets, solubilities, and expected bioavailabilities. Bioavailability, in turn, can be affected by a myriad of factors including source of TM, TM chemical characteristics and solubility (Miller et al., 2020), dietary characteristics such as dietary NDF (Kabaija and Smith, 1988), and levels of antagonists in the diet and gut environment (Overton and Yasui, 2014). The inverse is also true – differences in solubility of TM can modulate digestibility of other nutrients by affecting ruminal microflora (Caldera et al., 2019). Common forms of TM used to supplement cattle include sulfates, oxides, carbonates, and chlorides (Daniel et al., 2020). In the past, the livestock industry has relied mostly on oxide TM sources before shifting to sulfate TM sources, although more recently there is some evidence pointing to benefits of TM supplementation using more bioavailable inorganic or organic TM

sources (Overton and Yasui, 2014). Copper (Cu), Zinc (Zn) and Manganese (Mn) are the common trace minerals supplemented for dairy cows, deficiencies of which may lead to various health problems. Other TM considered important may already be present in sufficient quantities in ration not warranting supplementation. Copper is required for functioning of numerous enzymes and cofactors and its deficiency in cattle can lead to salt-sickness, severe diarrhea (teart), and bone disorders such as widening of epiphyses (Suttle, 2010). Similarly, Zn deficiency can result in severe parakeratosis, anorexia, inappetence, bone disorders and infertility in cattle (Miller and Miller, 1962; Pitts et al., 1966; Suttle, 2010). Mn deficiency has shown to reduce conception rates, cause delayed estrous and birthing of deformed calves e.g. fetlock abnormality colloquially called knuckle over (Corah, 1996).

Given the potential effects of TM like Cu, Zn, and Mn, solubility of different TM sources at various gastrointestinal sites may be an important consideration. According to Caldera et al. (2019), solubility of Cu and Zn hydroxy TM at a ruminal pH of 6.23 was lesser than that of sulfates of these minerals. Also, addition of Zn sulfate and Cu sulfate to a washed suspension of ruminal microorganisms reduced cellulose digestion, although Mn sulfate did not have an effect (Hubbert et al., 1958). In vitro, even minute quantities of Cu and Zn inhibited cellulose digestion (Hubbert et al., 1958). Less soluble TM sources in the rumen may result in decreased bioavailability of Zn and Cu to cellulolytic microorganisms (Daniel et al., 2020), potentially leading to lesser interference. Hence, the hypothesis of our meta-analysis is that hydroxide-based TM sources will lead to greater NDF digestibility, DM digestibility and DMI when compared to sulfates of Cu, Mn and Zn. The objective of the meta-analysis is to determine whether there is evidence in the literature that trace mineral sources of Cu, Zn and Mn affect NDF and DM digestibility and/or DMI.

MATERIAL AND METHODS

Literature search

Studies and reports, including published and unpublished reports, were considered for inclusion in the meta-analysis. Inclusion of unpublished studies in the meta-analysis may help to counter possible publication bias since there is evidence that research studies with nonsignificant results are less likely to be published (Sterling, 1959). Publication bias is a common problem that can significantly alter results of effects being investigated (Thornton and Lee, 2000). An attempt was made to ensure inclusion of all studies evaluating sulfate vs. hydroxy TM sources in the meta-analysis, by conducting a literature search between July and September 2021.

Google Scholar (<https://scholar.google.com/>) was searched using the following search terms: ('trace mineral source' AND 'sulphate' AND 'digestibility' AND hydroxide' OR 'intellibond'). Similarly, Scopus (<https://www.scopus.com/>) was searched using the terms ('trace AND mineral AND sources) AND ('dairy OR cows'). After exclusion of duplicate results, 78 studies (Figure 3.5) were obtained. Studies were considered for inclusion only if cattle were fed identical basal diets with sulfate and hydroxy TM added in equal quantities (mineral basis). Only studies containing at least some measure of digestibility while comparing the hydroxy and sulphate TM sources in cattle were retained.

To our knowledge, Micronutrients USA LLC (Indianapolis, IN) markets the only commercially available hydroxy TM available in the feed industry. Therefore, we also sought reports through this company. Of the 7 reports provided by Micronutrients, 4 were published in peer-reviewed journals, 2 were unpublished reports, and 1 was partially published.

After screening, 8 unique reports were found to meet criteria for inclusion in the meta-analysis. The 8 reports provided a total of 12 comparisons, shown in Table 3.1.

Data Extraction

The factors included to account for variability and possible effects on results included method of digestibility analysis, study design, beef vs. dairy cattle use, TM feeding rates, days on TM treatment, intake as % of BW, and % NDF in diet. Also included were the number of animals in control and treatment groups, standard error of the mean (SEM) for response variables, and the mean difference in the response variables (hydroxy TM mean – sulfate TM mean). For standardization, the 24-h time point was used for studies where *in situ* methods were used for digestibility assessment. One (Daniel et al., 2020) of the 4 studies categorized using the uNDF marker for digestibility assessment used a 336-h incubation to define undigested NDF, whereas the remaining 3 studies used a 240-h incubation. Although this difference may have led to trivial differences in absolute digestibility results, the relative differences between treatments within study were likely unaffected, and all uNDF marker studies were considered to have used a similar method.

When additional data on diets or other study factors was required, authors were contacted to request the data (n = 1). Only digestibilities of DM and NDF were available from all the studies, whereas ad libitum DMI response was available for 9 of the 12 comparisons. Studies and comparisons are listed in Table 3.1.

Statistical Analysis

Data were analyzed with a mixed-model approach using SAS software (v. 9.4), treating study as a random effect and other explanatory variables tested as fixed effects in assessing the mean impact of hydroxy TM. All results are defined as the response to hydroxy TM (hydroxy

mean – sulfate mean). Studies were weighted based on the inverse of the squared SEM for the outcome of interest. Funnel plots (JASP v. 0.12.2; Figures 3.1 - 3.4) were used to evaluate the potential for publication bias in the dataset, and the Test of Residual Heterogeneity (Q test) was used to assess whether substantial between-study variation in response existed. When the Q test was significant, predictive variables were tested in the model and retained when $P < 0.05$.

RESULTS

Dry Matter Digestibility

DM digestibility data was available from 12 treatment comparisons derived from a total of 268 individual animal observations. We found no evidence for an overall effect on DMD (+0.50 % units, SEM = 0.27, $P = 0.11$; Table 3.2) when hydroxy TM replaced sulfate TM. However, assessment of the model revealed significant heterogeneity ($Q = 22.49$, $P = 0.02$) across studies. After evaluating multiple explanatory factors, sector (beef vs. dairy) was retained as the only significant factor in the model ($P = 0.01$), and after accounting for sector, residual heterogeneity was no longer significant ($Q = 7.36$, $P = 0.69$). The results showed a significant mean increase of 1.64% units (SEM = 0.35, $P < 0.001$; Table 3.3) in DMD when hydroxy TM were consumed by beef animals. However, for dairy cattle a non-significant mean increase of 0.16% units (SEM = 0.13, $P = 0.29$; Table 3.3) in DMD was observed. The forest and funnel plots for the final DM digestibility model are shown in Figure 3.1. The funnel plot did not show any evidence of observation bias (Egger's test of asymmetry: $P = 0.96$), suggesting that a representative sampling of outcomes is included in the analysis.

NDF Digestibility

NDFD data from the 12 treatment comparisons derived from a total of 242 individual animal observations were included in the statistical model. Overall, NDFD increased significantly (+1.51% units, SEM = 0.49, $P = 0.02$; Table 3.2) in response to hydroxy TM. As in the case of DMD, however, model assessment again showed heterogeneity ($Q = 33.09$, $P < 0.001$) across studies. After testing all study factors in the model, digestibility assessment method was retained as the only significant factor in the final model ($P = 0.01$), and residual heterogeneity was not significant anymore ($Q = 6.65$, $P = 0.67$). Studies using the total collection method and uNDF as a marker showed a significant mean increase of 2.68 % units (SEM = 0.40, $P < 0.01$; Table 3.4) and 1.08 % units (SEM = 0.31, $P = 0.02$) in NDFD, respectively. However, studies using the 24-h in situ incubation method did not detect a significant change in NDFD (-0.03 % units, SEM = 0.23; $P = 0.89$).

We considered possible confounding of digestibility method and animal type in the evaluation of factors affecting NDFD, despite the failure of sector (beef vs. dairy) to be retained in the NDFD model. The greatest mean NDFD response was shown for total collection methods ($P < 0.04$ vs. other methods), which were used in 2 dairy and 3 beef animal studies, and in situ methods were used in 1 dairy and 2 beef studies. Use of uNDF as a digestibility marker was the only method that was not represented in both sectors, as it was used in dairy cattle studies ($n = 4$) and no beef studies. We found no evidence of digestibility method \times sector interaction in the model for NDFD ($P = 0.72$), suggesting that differences in outcomes were not due to confounding. Forest and funnel plots for the final NDFD model are shown in Figure 3.2. The funnel plot again revealed no evidence of observation bias (Egger's test of asymmetry: $P = 0.41$).

Dry Matter Intake

For DMI, 9 out of 12 available comparisons were used in the meta-analysis, as DMI was restricted to 90% of ad libitum intake in 3 of the studies. Overall, DMI per animal (kg/d) and DMI per unit bodyweight (%BW) showed a non-significant mean response of +0.30 kg/d (SEM = 0.35, $P = 0.43$; Table 3.2) and +0.04 % BW (SEM = 0.048, $P = 0.47$), respectively.

Heterogeneity was observed for this variable when expressed both in kg/d ($Q = 26.29$, $P < 0.001$) and as a % of BW ($Q = 208.64$, $P < 0.001$), as shown in Table 3.2. However, no additional factors were retained in the model, so the response heterogeneity was not resolved with the data available. The distribution of responses for DMI metrics are represented in forest and funnel plots (Figures. 3.3 and 3.4), which did not suggest bias in the data.

DISCUSSION

Hydroxide-based TM significantly increased DMD in beef cattle but not in dairy cattle studies. The difference in DMD across sectors was not explained away by variation in diet type, DMI (% of BW) or NDF content of the diets. So, there is little evidence to suggest that an interaction of TM source with either passage rate or dietary fiber content explains the observed different responses between beef and dairy cattle models. Hence, rather than diet type, DMD response may be better explained by inherent physiological and possibly anatomic gastrointestinal differences, especially considering all the dairy cows in the meta-analysis were lactating. Trace mineral source can affect ruminal fermentation (Faulkner and Weiss, 2017) and different microbiota of dairy vs. beef cattle may contribute to these different responses; unfortunately, studies directly comparing ruminal microflora of beef and dairy animals in relation to nutrient digestibility are rare. However, it was demonstrated by (Weimer et al., 2010)

that dairy cows can reestablish ruminal bacterial community composition (BCC) with some success post significant ruminal microflora challenge from another dairy cow. Although not a direct comparison between beef and dairy animals this shows that host specificity of ruminal BCC is characteristic even among different animals within a given sector. Given that it can reasonably be assumed that the variation in ruminal BCC of beef cattle would be far greater when compared to dairy cattle hence the potentially explaining the observed variation in DMD responses.

Like comparative ruminal microflora research work, studies comparing gastrointestinal phenotype and nutrient digestibilities between beef and dairy animals are rare. While comparing different dairy cow genotypes (Beecher et al., 2014) found an increased significant effect of cow genotype on DMD, NDFD and ADF digestibility when comparing Jersey with Holstein cows. Relatively larger size of GIT to body weight in Jerseys, increased grazing frequency and ruminating mastication (Prendiville et al., 2010) might help explain the variation in nutrient digestibilities in this case. Likewise, it can be reasoned that physiological and gastrointestinal properties of beef steers are markedly different from lactating dairy cows. Also, a phenotypical comparison of Holstein and Charolais bull organs (Pfuhl et al., 2007) revealed that stomachs (rumen, omasum and abomasum) percentage in relation to empty body weight were significantly higher for Holsteins. Plus, both small and large intestines weight as a percentage of empty bodyweight were higher for Holstein bulls. If heavier GIT in Holsteins is any measure of its length, perhaps it can be speculated that at similar or even lower passage rates, hydroxide TM would be less bioavailable throughout the GIT of beef animals thereby lesser interference of metals with gut microflora leading to increased DMD. The observed differences for DMD between dairy and beef animal might be due to any one or a collective function of reasons and

extrapolations discussed here. Probably the best method of achieving more conclusive results will be to increase the number of both beef and dairy comparisons for the analytical methods.

While there was a slight overall effect of hydroxy-based TM on DMD, an overall significant increase of 1.51% (SEM = 0.49; $P = 0.02$; Table 3.2) in NDFD was evident for hydroxy-based minerals supplementation. Heterogeneity across studies for NDFD revealed digestibility assessment method ($P < 0.001$) as the only significant factor in the model. Studies using total collection method (+2.68%; SEM = 0.54; $P < 0.001$) and uNDF (+1.082%; SEM = 0.31; $P = 0.02$) showed significant improvement in NDFD when hydroxide TM were used, whereas studies employing 24-h *in situ* incubation (-0.034%; SEM = 0.23; $P = 0.89$) showed little change. Variation in NDFD changes observed across studies using the 3 different methods can be reasonably expected. Interestingly, results from some studies (Faulkner and Weiss, 2017; Caldera et al., 2019; Miller et al., 2020) indicate increased total tract digestibility of macronutrients like NDF while others (Genther and Hansen, 2015) showed little improvement in NDFD when supplementing animals with hydroxy TM. Total fecal collection is the gold standard procedure (Lee and Hristov, 2013) for accurate nutrient digestibility assessment. The fact it showed a clearly significant impact of TM source indicates that the effect observed for NDFD is likely real.

However, total collection methods can be impractical and labor intensive depending on the number of animals involved, and intrinsic or extrinsic markers have been used as an alternative approach to estimating fecal output, and similar results between total collection and uNDF-based methods here suggest reasonable agreement in terms of impacts of TM source on total tract digestibility. However, significant interactions between intrinsic markers and diets are not uncommon (Lee and Hristov, 2013; Daniel et al., 2020). In our analysis, uNDF methodology

was confounded with animal type, as all 4 studies using this method were dairy animal studies, which may have contributed to the observed mean. However, method by animal type interaction was not evident in the model for NDFD ($P = 0.72$), and animal type was not retained. Inoculum donor effects (Fahey et al., 1994) can affect the difference in magnitude of NDFD response observed when comparing uNDF methods to total collection.

In situ incubation failed to yield similar results, which can perhaps be attributed to the models being noisy, making it harder to detect relatively small differences in NDFD. Adding to the complexity of using 24-h *in situ* incubation are potential factors affecting *in vivo* nutrient digestibility (e.g., diet composition). According to Firkins (1997), typical rations having NDF content of 25-30% with a higher ratio of potentially digestible fiber will result in increased hindgut fermentation of potentially digestible fiber. Moreover, it has been demonstrated that forage processing, maturity and inclusion of rapidly fermenting carbohydrates can vary the proportion of cellulose and hemicellulose digestion in the hindgut (Hoover, 1978). Thus *in-situ* methods may not be able to capture these altered hind gut fiber digestibility changes. Other factors affecting 24-h *in situ* accuracy are methods materials used e.g., *in-situ* bag location within the rumen, rinsing technique used (Fahey et al., 1994) pore size, basal diet effect on microbial populations migrating inside the bag (Meyer and Mackie, 1986). Therefore, there is a real possibility that 24-h *in situ* will not completely and accurately capture the effect of TM source on NDFD when compared to total collection method both, ruminally and post-ruminally.

Neither metric of DMI was significantly affected by TM source (Table 3.2). This can possibly be a result of inability of gut microflora to adapt to hydroxy or sulfate TM within days on treatment. Usually a 14-d adaptation period is considered adequate for changeover and crossover experiments (Machado et al., 2016), although a range of 7-27 day adaptations are not

unheard of for digestibility trials. These ranges of days for adaption may be appropriate for macronutrients like NDF or starch which can affect treatment responses significantly and form bulk of ration. However, in case of TM perhaps much longer days on treatment might be required to be able to detect a treatment response. Extent of TM solubility determines the concentration of metallic minerals exposed to rumen microbes, which may be an important factor determining its interaction with microbes (Genther and Hansen, 2015). For example, Cu from hydroxy-based TM was found to be less soluble and available in the rumen for fiber interactions but similar solubility as sulfates in the acidic abomasum (Genther and Hansen, 2015). Cu is known to be toxic to cellulolytic bacteria even in small quantities. This selective solubility depending on gut compartment environment might be one way hydroxy-based TM can differ from sulfates. However, in the meta-analysis only one dairy study (Trouw Burfod) fed the mineral supplements for 32 days which was the maximum number of days on treatment compared to other studies. According to Genther and Hansen (2014) TM profile of beef steers was a critical factor during disease or shipping stressors in maintaining DMI. In a separate beef steer TM supplementation level study by Niedermayer et al. (2018), no mean difference in DMI (kg/d) on days 0-56 was reported, however inorganic TM supplementation from days 56-124 increased DMI when compared with control, though not among varying levels of TM supplementation. This indicates perhaps long-term studies using different TM sources might yield more conclusive results for DMI.

Another indirect factor that could possibly affect DMI is the effect of TM source on DMD or NDFD. It has been suggested by Oba and Allen (1999) that increased NDFD was positively associated with DMI and milk yield in dairy cows. Increased DMI in lactating cows leads to higher rate of passage of digesta, which can depress nutrient digestibility (Tyrrell and

Moe, 1975; de Souza et al., 2018). This could possibly mask any DMI effect by TM for studies involving lactating cows. Given the limited number of comparisons supplementing TM, additional comparisons may be necessary for reliable interpretation of DMI treatment responses.

CONCLUSION

Hydroxy TM compared to sulfates positively impacted DMD in beef but not in dairy animals. Hydroxy TM also showed an overall increase in NDFD; however, digestibility assessment method affected response, with largest impact observed for total collection studies followed by those using uNDF as a marker, where those using 24-h in situ methods did not detect any change in NDFD. Since total collection method is widely considered gold standard for measuring digestibility, the results give credence to hydroxide TM the response. Overall, response due to different TM source maybe dependent on cattle type, digestibility assessment methods and properties of the mineral source itself and how it interacts in the rumen and within the animal in general.

APPENDIX

Table 3.1. List of studies, comparisons and explanatory variables included in the meta-analysis.

Reference	Study Type	Design code	Method	Top Dress or TMR	Beef or Dairy
Genther and Hansen, 2015. (Low level)	5×5 Latin square	LS	In situ 24-h	TMR	Beef
Genther and Hansen, 2015. (High level)	5×5 Latin square	LS	In situ 24-h	TMR	Beef
Micronutrients Trial #2017D103CACZM	Split-plot replicated Latin square	LS	In situ 24-h	TMR	Dairy
Miller et al., 2020. (conventional)	Replicated 4×4 Latin square	LS	uNDF ₂₄₀	TMR	Dairy
Miller et al., 2020. (brown mid rib)	Replicated 4×4 Latin square	LS	uNDF ₂₄₀	TMR	Dairy
Faulkner and Weiss, 2017. (Byproduct)	Split-plot replicated Latin square	LS	Total collection	Top dress	Dairy
Faulkner and Weiss, 2017. (Forage)	Split-plot replicated Latin square	LS	Total collection	Top dress	Dairy
Caldera et al., 2019.	Randomized block	RCBD	Total collection	Top dress	Beef
Guimaraes et al., 2020. (Low Fiber, Dairy)	Randomized block	RCBD	Total collection	TMR	Beef
Guimaraes et al., 2021. (High Fiber)	Randomized block	RCBD	Total collection	TMR	Beef
Penn State Micronutrients trial Nov18	Crossover	LS	uNDF ₂₄₀	TMR	Dairy
Daniel et al., 2020.	Latin square	LS	uNDF ₃₃₆ *	TMR	Dairy

Table 3.2. Simple least-squares mean estimates of responses to replacing sulfate TM with hydroxy TM and test of response heterogeneity.

Outcome	Comparisons (n)	Mean response	SEM	P value (Trt Means)	Q	P value (Q)
DM digestibility (%)	12	+0.50	0.27	0.11	22.5	0.02
NDF digestibility (%)	12	+1.51	0.49	0.02	33.1	< 0.001
DM intake (kg/d)	9	+0.30	0.35	0.43	26.3	< 0.001
DM intake (% of BW)	9	+0.04	0.048	0.47	208.6	< 0.001

Table 3.3. DM digestibility (%) response by animal type for hydroxy TM vs. sulfate TM sources.

Sector	Comparisons (n)	Mean response	SEM	P value
Beef	5	+1.64	0.35	< 0.001
Dairy	7	+0.16	0.13	0.29

Table 3.4. NDF digestibility (%) response by digestibility assessment method for hydroxy TM vs. sulfate TM sources.

Method	Comparisons (n)	Mean response	SEM	P value
24-h <i>in situ</i>	3	-0.03	0.23	0.89
Total collection	5	+2.68	0.40	< 0.01
uNDF marker*	4	+1.08	0.31	0.02

*One of the studies used uNDF₃₃₆ while the other 3 used uNDF₂₄₀ as the marker method.

Figure 3.1. Forest plot and funnel plot for DM digestibility (%) response by animal type for hydroxy TM vs. sulfate TM sources. Individual study means are represented by circles with the sub-category (beef and dairy) means shown by diamonds; error bars show the 95% confidence intervals.

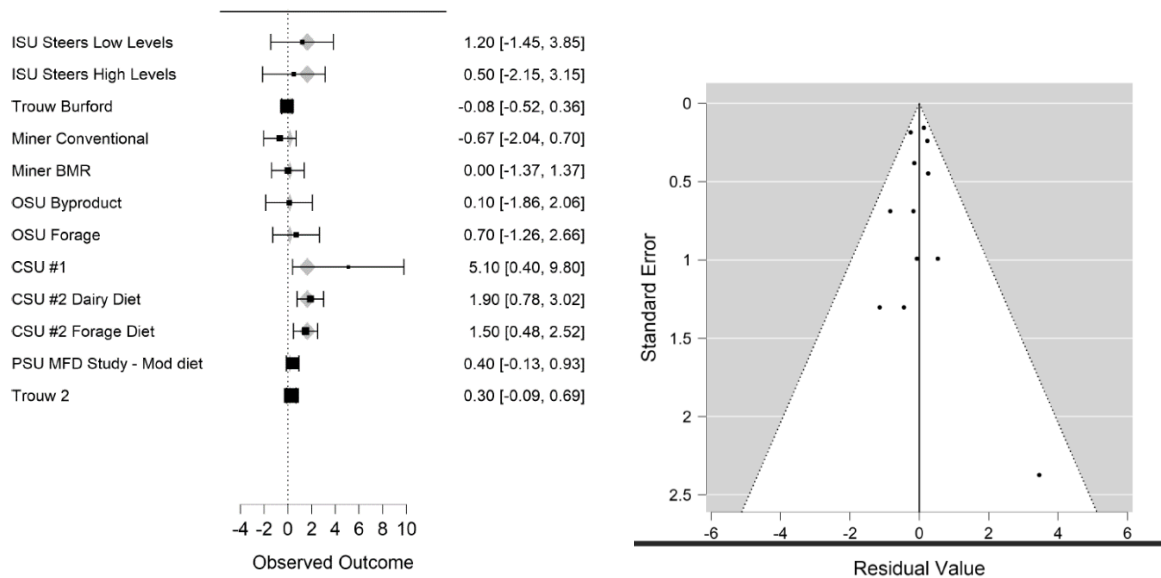


Figure 3.2. Forest plot and funnel plot for NDF digestibility (%) response by digestibility method for hydroxy TM vs. sulfate TM sources. Individual study means are represented by circles with the sub-category (digestibility method) means shown by diamonds; error bars show the 95% confidence intervals.

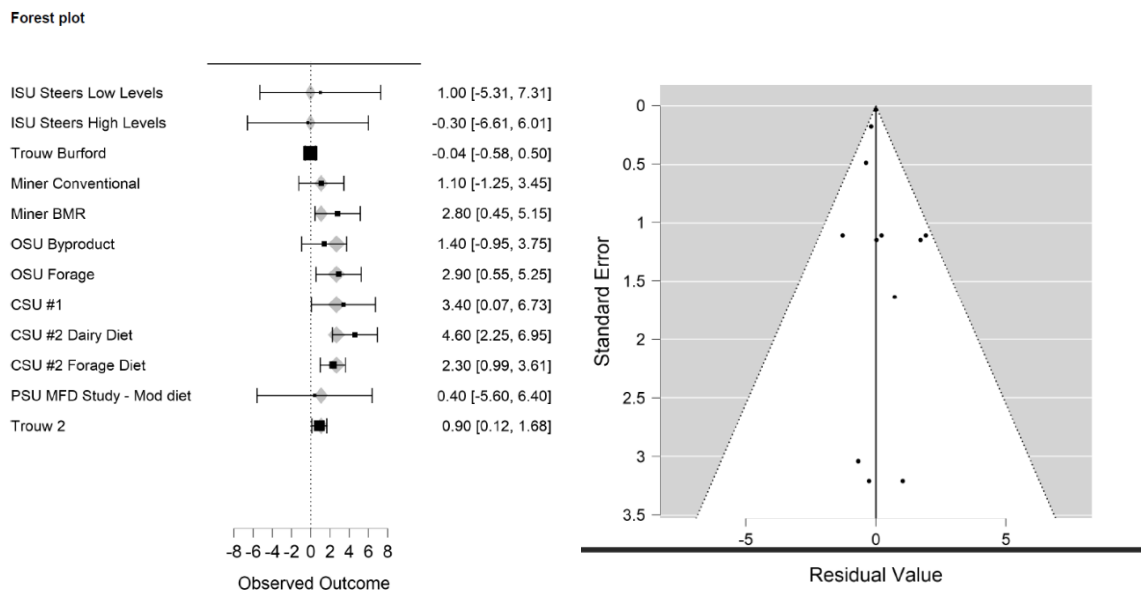


Figure 3.3. Forest plot and funnel plot for DM intake (kg/d) response for IntelliBond vs. sulfate mineral sources. Individual study means are represented by circles with the overall mean shown by the diamond; error bars show the 95% confidence intervals.

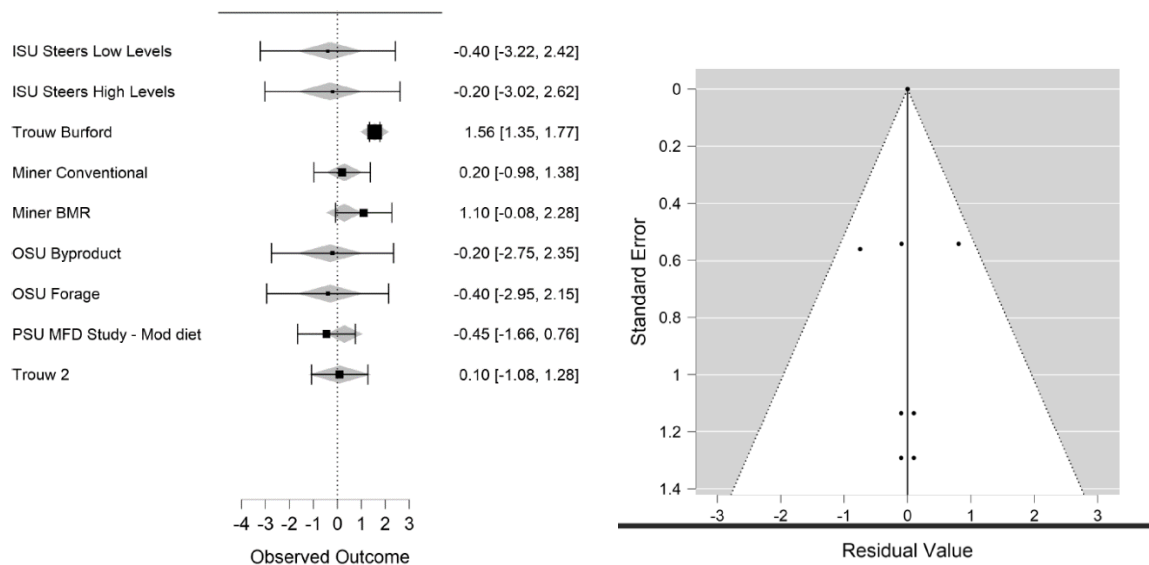


Figure 3.4. Forest plot and funnel plot for DM intake (% of body weight) response for IntelliBond vs. sulfate mineral sources. Individual study means are represented by circles with the overall mean shown by the diamond; error bars show the 95% confidence intervals.

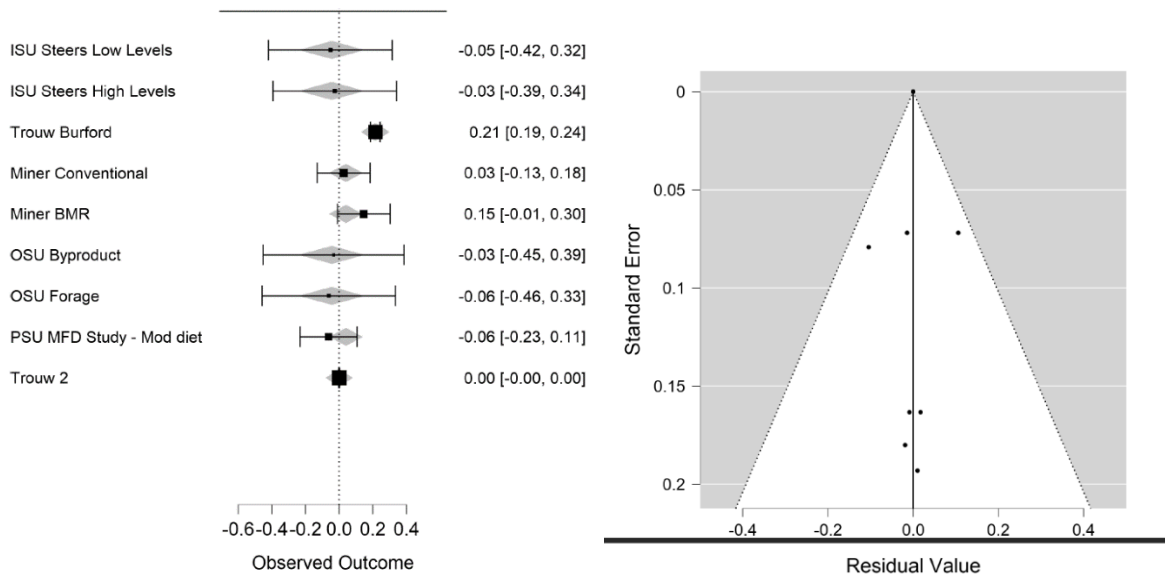
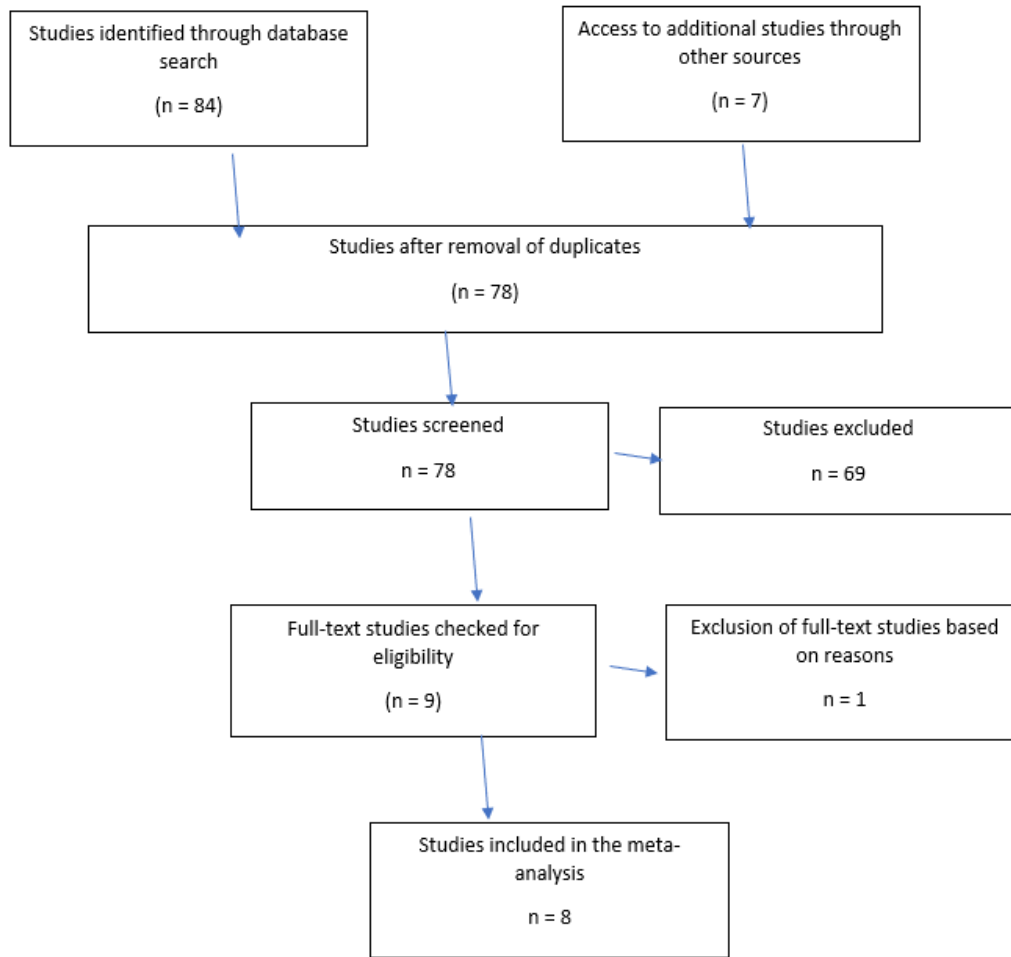


Figure 3.5. PRISMA (Preferred Reporting Items for Systemic Reviews and Meta-Analysis) flow diagram showing the stages from initial search to finalization of publications included in the meta-analysis evaluating effects of TM source supplementation on DMD, NDFD and DMI.



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Chapter 4 – Overall Conclusions

Strategies geared towards increasing fiber digestibility in dairy cattle diets can improve milk yields due to greater nutrient availability and DM intake, possibly resulting in increased farm profitability. Improved fiber digestibility can be achieved by use of more digestible forages or feedstuffs as well as inclusion of mineral sources that ultimately lead to improved digestibilities. Similar to BMR corn, development of high quality or lower lignin and reduced-lignin alfalfa varieties was aimed at increased in vivo fiber digestibility of hay. The objectives of the alfalfa study (chapter 2) were to determine whether lactating diets fed at 50% forage, 2/3 of which came from low-lignin (LL), a blend (BLD) or conventional alfalfa hay (CON) would affect DMI, nutrient digestibility, or production of milk and milk components. Secondly, the objective of the meta-analysis (chapter 3) was to determine whether the literature indicates any differences in NDFD, DMD and DMI based on trace mineral source, comparing sulphates to hydroxide TM. Together these studies evaluated the effects of alfalfa hay variety and trace mineral source on digestibility, particularly emphasizing fiber digestion.

In chapter 2, LL hay did not affect DMI, milk yield or components except for a dose-dependent decrease in milk fat concentration. Contrary to our hypothesis, the CON treatment showed greater NDFD and milk fat concentration compared to LL, although fat yield did not change. Thus, the results indicate that small differences in dietary aNDFom (lesser for LL) plus somewhat lesser digestibility of CON alfalfa may have resulted in greater physically effective fiber for CON treatment, promoting improved ruminal function. This may also be a result of greater particle break down for LL, leading to greater passage rate and secondary effects on rumen pH. Hence, keeping a greater safety margin for peNDF while formulating using low-lignin alfalfa hay is advised. We believe this study can serve as a basis for designing future

experiments investigating the effects of low and/ or reduced-lignin alfalfa in lactating animals. In future research, use of equal NDF diets with careful consideration for peNDF may further add value to findings. Perhaps experimental use of alfalfa hay from multiple plots from across the country can better contribute to our overall understanding of LL alfalfa in dairy cattle nutrition.

In chapter 3, the meta-analysis including 8 studies and 12 comparisons revealed that hydroxy TM improved DMD in beef but not in dairy animals compared to sulphate TM. Also, these differences were not explained simply by dietary differences, DMI (% of BW), or NDF content, and may be a result of inherent physiological differences between beef and dairy cattle. Hydroxy TM caused an overall increase in NDFD, although digestibility assessment method affected this response. The greatest increase in NDFD was observed for total collection methods, widely considered the gold standard for measuring nutrient digestibility, thus giving some credence to our findings. No change in DMI intake was found. Overall, variation in response due to TM source may depend on cattle type, digestibility assessment method and an effect on ruminal environment due to interactions. Given the limited number of studies available for the analysis, additional studies and comparisons between sulphate and hydroxy TM can further improve the reliability of our findings.

Together, chapters 2 and 3 highlight apparently different dietary interventions that can modulate nutrient digestibility - especially NDFD - in lactating cows. The effects of different dietary interventions on nutrient digestibility and production parameters may or may not be additive. However, our findings show the potential and important considerations for dietary interventions for improving nutrient digestibility along with production parameters.