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Milk Fat Depression: Etiology, Theories, and Soluble Carbohydrate Interactions

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Abstract

Milk fat depression is a metabolic disorder characterized by a sustained reduction in milk fat and has challenged producers and scientists for over a century. Nutritionists have incorporated supplemental fat in dairy rations for years as a means to increase the energy density of the diet, but often decreased fiber digestibility or milk fat depression was observed. The rumen microbial populations were thought to play a major part in why producers had difficulty using high levels of unsaturated fat. Many different theories evolved throughout the century into the causes of milk fat depression that often appears ex nihilo. Such theories involved concepts centered on substrate limitation, low rumen pH, and specific bioactive fatty acids. The biohydrogenation theory became one of the more widely accepted explanations for milk fat depression. Since the discovery of certain fatty acid isomers that induce milk fat depression more investigations have aimed at how different nutrients interact in the rumen and affect the production of these milk fat inhibiting isomers. Readily available carbohydrates such as sugar, starch, neutral detergent soluble fiber, and rumen availability of these carbohydrate fractions, all effect the microbial populations within the rumen that are involved in the process of biohydrogenation. This review summarizes the knowledge available on milk fat depression, microbial influences, how soluble carbohydrate fractions influence biohydrogenation.

Keywords Milk fat depression; Starch; Soluble fiber; Biohydrogenation; *In vivo*; *In vitro*

Introduction

Inclusion of lipids into lactating dairy cow diets is a challenge due to the demand to meet the needs of the animal as well as the complexity of the rumen. Milk fat depression (MFD) is a centuries-old metabolic syndrome characterized by sustained drops in milk fat percentage. Milk fat is highly sensitive to changes in diet, and has been extensively investigated in ruminants. In many species, the fatty acid composition of milk fat reflects the fatty acid profile of the diet [1]. However, ruminants are unique in that the lipids consumed, depending on physical and chemical properties, will be transformed by the bacterial metabolism in the rumen. Diet can alter the bacterial populations and rumen fermentation, which can have major effects on the fat content and fatty acid composition of milk.

Dietary, metabolic, and microbial changes due to milk fat depression have been investigated thoroughly and great strides have been made in the last 20 years in determining risk factors for the onset of the disorder. Recent work by Rico et al. [2] characterized the time course of induction and recovery from MFD, allowing researchers to have a greater understanding of the time required for MFD to emerge as well as how long it will take for farmers to recover milk fat to normal levels. Elucidating the onset of MFD and dietary modifications that can be made to recover milk fat is paramount to better understand the multitude of factors that may contribute to the disorder. Microbial population changes during MFD have been well documented, with these alterations being highly sensitive to changes in the ruminal synchrony of nutrients. Some research has focused on the utilization of sugars, neutral detergent fiber-rich byproducts, and processed grains to increase the productivity of lactating cows [3-5]. However, some of these investigations observed some interesting effects related to milk fat production. For example, Martel et al. [4] reported that biohydrogenation was enhanced and rumen pH was increased with the inclusion of molasses in the diets of lactating dairy cows fed high-energy diets. There is evidence that sugar may decrease proportions of polyunsaturated fatty acids (PUFA) and *trans* 18:1 fatty acids in milk fat and increase milk fat content through the modulation of ruminal pH and fatty acid biohydrogenation pathways [6-8]. Sources high in neutral detergent soluble fiber have also been utilized for their economic benefits as well as their positive impacts on rumen fermentation. With the demand for corn driving the use of byproducts, research on feedstuffs such as soy hulls, citrus pulp, and beet pulp has increased. Voelker et al. [9] replaced a portion of the corn with beet pulp and observed a linear increase in milk fat. The soluble fiber in these byproducts are a source of rapidly fermentable energy that has been reported to yield more acetate and increase proliferation of cellulolytic microbiota, which may favor increased biohydrogenation and reduction of *trans* intermediate production in MFD scenarios [10]. Replacing a portion of the starch with sugars or beet pulp may improve production, but the degradability of the starch fraction is an important factor to consider as well. The structural organization

of corn is likely the cause of decreased digestibility of whole shelled corn, and attempts have been made in order to increase starch availability, such as steam flaking. Ground high moisture corn and steam flaked corn have been reported to increase milk yield and protein percentage [11]. However, there has been some evidence that increased starch degradability yields greater proportions of *trans* intermediates [12,13]. Therefore, it is of interest to investigate ruminal and whole-animal responses to changes in dietary sugar, starch degradability, and neutral detergent soluble fiber with respect to preventing or ameliorating milk fat depression.

Fat in Ruminant Diets

Fat is commonly added to rations as a means to increase the energy density of the diet, improve palatability and reduce feed dust, and more recently, to alter the fatty acid (FA) profile of the meat or milk end product [14]. However, it is widely accepted that not all fats are created equal with regards to ruminants. Grass and corn silages contain 1-3% total FA, and grains and by-products vary in their FA content depending on processing [15]. The amount of FA within plant species is highly dependent upon maturity at harvest, season, environment, and a variety of other factors. Silages and preserved forages make up a significant part of a dairy cow ration and are commonly added along with commercial fat supplements.

Triglycerides, galactolipids, and phospholipids are the three predominant glycerol-based lipids in animal feed ingredients [16]. Galactolipids make up a major portion of the glycolipids within forages and consist of a glycerol backbone with a bound galactose molecule and two FA [17]. Triglycerides are the major storage lipid in oilseeds, and therefore are greater in concentration in concentrate feedstuffs. Addition of fat to rations is common practice in order to increase dietary energy density without the risk of feeding excessive amounts of fermentable carbohydrates [18]. However, when added to a ration at a high inclusion rate, supplemental fat can have negative effects on dry matter intake, milk yield, and milk components [19].

The ideal fat is one that has no effect on rumen fermentation, has high intestinal digestibility, is easy to handle and mix into the total mixed ration (TMR), and increases milk and milk component yields. This is an inherent problem with unsaturated fatty acids, as they are highly modified by microbiota once they enter the rumen, so what goes in the system might not be the same post-rumen. Attempts to protect unsaturated fatty acids from microbial action have been developed by forming calcium salts, which minimize ruminal transformation of unsaturated fatty acids and risk of a decrease in fiber digestibility [16]. The discovery of these calcium salts could be considered somewhat of an accident. In an experiment conducted in the late 1970s by Dr. Don Palmquist the animals experienced a bout of milk fever that required the supplementation of calcium to alleviate the problem [20]. After analyzing the data it appeared that adding calcium to the diets improved the utilization of fat as well as the entire ration, thus beginning the investigation into developing calcium salts of fatty acids. These calcium salts are considered “bypass” or “rumen inert” fats and are formed by a reaction of divalent cations of Ca with long chain fatty acids (LCFA) to form insoluble fatty acid complexes [21]. Many of the commercially available Ca-salt FA supplements are derived from palm FA distillate and contain both saturated and unsaturated FA [22]. Some other ways of protecting fatty acids include encapsulation and hydrogenation. Natural encapsulation of fatty acids is also a form of protection in certain feedstuffs such as whole oilseeds, as the fatty acids are protected by the tough seed coat. However, disruption of this seed coat by mastication or disruption in the feed mixer can lead to the fatty acids being exposed and increases the risk of microbial transformation.

Mohamed et al. [23] reported that milk fat percentage was reduced when soybean or cottonseed oil were included in the diet, whereas whole cottonseed or whole soybeans did not reduce milk fat. This suggests that feeding whole oilseeds are less likely to lead to the onset of diet-induced MFD as compared to feeding free FA as oils. Other forms of encapsulation have been reported in the literature and include physical and chemical modifications. For example, encapsulation by combining unsaturated FA with casein and rendering it inert by cross-linking with formaldehyde or encasing the unsaturated FA within a saturated FA shell [24].

Saturated fats are a popular choice among dairy producers and nutritionists due to their versatility in how they can be added to rations without risk of causing milk fat depression. Saturated fatty acids have been shown to positively affect milk yield without decreasing dry matter intake and also have earlier resumption of ovarian activity, resulting in decreased days open [25]. Two of the most common saturated fatty acids in many fat products are palmitic (C16:0) and stearic (C18:0) acid. Although these fatty acids differ only in two carbons, they have unique and specific roles in the dairy cow. Palmitic acid is found in greater quantities in adipose tissue, whereas stearic acid is the predominant fatty acid absorbed by the mammary gland in the lactating dairy cow [26]. Levels of C18:0 are greater at the intestinal level than what was originally fed due to complete biohydrogenation of unsaturated fatty acids within the feed. Feeding saturated or unsaturated FA can supply necessary energy to the animal, but even excess saturated FA has been reported to depress fiber digestibility [18,27].

Rico et al. [19] investigated the effects of feeding a high-C16:0 or Ca-FA supplement in lactating cows and found that the supplement high in C16:0 increased DMI, milk yield, milk fat, and energy corrected milk (ECM) in comparison to the Ca-FA supplement that was higher in unsaturated fatty acids. However, reports of increased DMI or milk yields with feeding palmitic or stearic acid have been variable in some investigations [28,29]. Dietary FA profiles have a direct influence on milk fat concentration and yield. For example, abomasal infusion of short and medium chain saturated fatty acids increased the milk fat concentration and yield [30]. Strategic feeding of saturated FA for increased milk yields and energy density of the diet have also been under scrutiny for the impacts on milk products and consumer acceptability. A review by Livingstone et al. [31] suggests that consumption of dairy products where saturated FA were replaced with PUFA or MUFA improved plasma lipid markers of cardiovascular disease risk in humans.

Polyunsaturated FA, particularly of the n-3 family is of interest in human nutrition lately, due to the potential health benefits. However, the incorporation of PUFA or MUFA into milk fat is confounded by the complexity of nutrient interactions in the rumen as well as desaturation that occurs within the mammary gland. For example most of the *cis*-9, *trans*-11 conjugated linoleic acid (CLA) found in milk is primarily derived from the desaturation of *trans*-11 18:1 in the mammary gland itself [32]. The synthesis of milk fat and the origin of milk fatty acids will be discussed further in the next section.

Origin of Milk Fat

In order to understand dysfunction of mammary lipid synthesis, it is important to first understand the composition of milk and physiology of the mammary gland. It has been reported that milk contains over 400 fatty acids, of which 98% of the lipid is in triacylglycerol (TAG) form with three fatty acids esterified onto a glycerol-3-phosphate backbone [33]. Fatty acids within bovine milk fat are derived from two sources: they are synthesized *de novo* by the mammary gland, or are incorporated into the milk fat globule from plasma lipids originating from the feed or mobilization of body fat. *De novo* and preformed fatty acids differ significantly, with the *de novo* fatty acids ranging 4:0 to 14:0 in chain length, and preformed consisting of FA of greater than 16 carbons **(Figure 1.1)** [34]. Fatty acids of 16 carbons originate from both sources, as demonstrated by Palmquist et al. [35], who fed or intravenously infused $1^{-14}C$ palmitic acid into lactating cows. *De novo* fatty acid synthesis has been reported to contribute approximately 45% of the total fatty acids in milk [36].

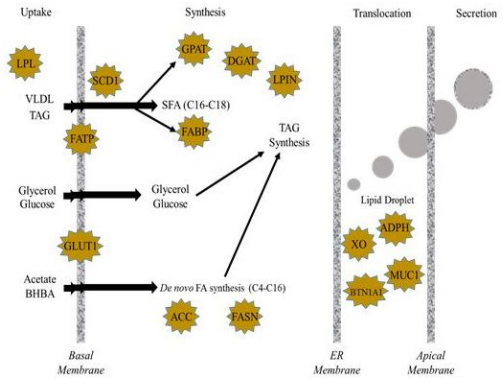

Figure 1.1: Coordinated activities and pathways of milk fat synthesis and secretion. Lipoprotein lipase (LPL); stearoyl-CoA desaturase (SCD); fatty acid transport proteins (FATP); glucose transporters (GLUT); glycerol phosphate acyltransferase (GPAT); diacylglycerol acyltransferase (DGAT); lipin (LPIN); fatty acid binding proteins (FABP); acetyl-CoA carboxylase (ACC); fatty acid synthase (FASN); mucin 1 (MUC1); butyrophilin (BTN1A1); xanthine oxidoreductase (XO); adiphophilin (ADPH); FA=fatty acid; UFA=unsaturated fatty acid; SFA=saturated fatty acid; VLDL=very low-density lipoprotein; TAG=triacylglycerol and BHBA=B-hydroxybutyrate. Adapted from McGuire et al. [37].

De novo fatty acid synthesis in the mammary gland utilizes mainly acetate and some β-hydroxybutyrate derived from rumen microbial fermentation **(Figure 1.1)**. Once in the mammary gland, acetate is activated to acetyl-CoA by acyl-CoA synthetase short-chain family (ACSS), then carboxylated to malonyl-CoA, which is catalyzed by acetyl-CoA carboxylase (ACC) [38]. Malonyl-CoA then undergoes a chain elongation process by addition of two CH_2 groups at a time catalyzed by the enzyme complex, fatty acid synthase (FAS) [39,40]. These fatty acids are straight-chain and possess an even number of carbons. However, propionate, valerate or isobutyrate can be utilized for fatty acid synthesis and result in the formation of branched-chain or odd-numbered carbon fatty acids [41]. The process of *de novo* synthesis requires reducing equivalents of nicotinamide adenine dinucleotide phosphate (NADPH), which are generated from the pentose phosphate cycle and the oxidation of isocitrate by isocitrate dehydrogenase [38].

The TAG-rich chylomicrons and VLDL of plasma are the primary source of preformed fatty acids taken up by the mammary gland, a process which is mediated by lipoprotein lipase [40]. Nonesterified fatty acids (NEFA) bound to albumin that is derived from the GI tract or mobilizations of body fat stores have also been reported to be a source of preformed FA [42]. The contribution of FA in milk from mobilized fat stores accounts for less than 10% of the FA in milk, but during periods where cows have a negative energy balance the contribution increases [34]. Fatty acids can also be desaturated within the mammary gland by stearoyl-CoA desaturase (SCD), which plays an important role in the modulation of unsaturation of membranes and TAG composition [38]. Palmitoleoyl-CoA, oleyl-CoA, *cis*-9, *trans*-11 and *trans*-7, *cis*-9 CLA isomers have been reported to be derived in part from SCD activity [32,42-45].

The TAG in milk is synthesized within the endoplasmic reticulum of the mammary epithelial cells by a variety of enzyme complexes. Fatty acyl-CoAs from *de novo* synthesis as well as preformed FA are esterified to a glycerol-3-phosphate backbone, which is derived from glycolysis or, less frequently, by phosphorylation of free glycerol by glycerol kinase [46]. Fatty acids are not distributed randomly on the sn-1, sn-2, and sn-3 positions on the glycerol backbone, which allows for FA to illicit functional or nutritional activities. Approximately half of the FA esterified at sn-1 and sn-2 are medium and long-chain saturated FA, and 44% of the FA at the sn-3 position is short-chain FA or oleic acid (27%). Fatty acids are esterified at the sn-1 position first by glycerol-3-phosphate acyl transferase (GPAT), which serves as the first committed step in TAG biosynthesis. Lysophosphatidic acid acyltransferase (LPAAT) catalyzes the second step with a greater preference for saturated FA (preference in order of C16>C14>C12>C10>C8) at the sn-2 position. Diacylglycerol acyltransferase (DGAT) esterifies both short and long chain fatty acids at the sn-3 position, and seems to be an enzyme complex unique to TAG synthesis [47]. The composition of milk fat can be modified by a variety of means including dietary changes, season, and physiological state. Milk fat from cows with milk fat depression has an altered FA composition, which will be discussed further in a later section.

Microbial Metabolism of Fats

The microorganisms within the rumen are responsible for a multitude of symbiotic functions to the animal. Unsaturated fatty acids are considered antimicrobial agents in that they incorporate into the bacterial cell and can disrupt its activity [48]. Many of the feeds that a cow consumes consist of naturally occurring unsaturated plant lipids that the rumen microbiome must saturate. Rumen bacteria utilize a defense mechanism that allows them to convert PUFA to saturated fatty acids. Unsaturated fatty acids enter the rumen and undergo two important transformations elicited by ruminal microorganisms: hydrolysis and biohydrogenation. Hydrolysis is the separation of FFA from ester linkages by microbial lipases **(Figure 1.2)**, which are extracellular enzymes packaged in membranous particles [41]. The released fatty acids then undergo biohydrogenation, a process which requires a free carboxyl group for its initial step [15].

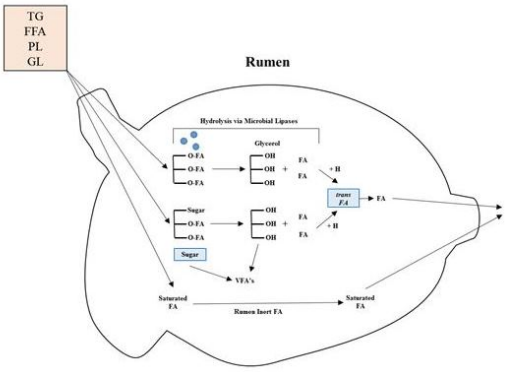

Figure 1.2: Lipid metabolism in the rumen. TG=Triglyceride, FA=Fatty Acid, PL=Phospholipid, GL=Glycolipid, FFA=Free Fatty Acid. Adapted from Davis [49].

Biohydrogenation is a process bacteria use to add hydrogen's to unsaturated fatty acids in order to saturate them. This protective mechanism is one of the primary reasons why the amounts of unsaturated fatty acids that enter the rumen do not equal the same quantity post-ruminally. There is a small amount of saturated FA that is incorporated into microbial membranes, but no fermentation of FA occurs.

The rate and extent of biohydrogenation is dependent upon rumen conditions such as pH and microbial populations. Instances such as low pH can result in incomplete biohydrogenation, leading to increased production of *trans* fatty acids [16]. Biohydrogenation of unsaturated fatty acids by ruminal microorganisms pose a specific problem for unsaturated fatty acid incorporation into tissues and milk. The mechanism of biohydrogenation could be considered a defense mechanism in that it enables unsaturated fatty acid-sensitive bacteria a way to tolerate the natural unsaturated fatty acids within plant tissues. Other factors such as total unsaturated fatty acid load within the rumen can influence the rate and extent of biohydrogenation and alter what fatty acid intermediates reach the small intestine. For example, fish oil supplementation alone or in combination with unsaturated plant oils have been shown to increase the amount of biohydrogenation intermediates escaping the rumen, particularly those with *trans* double bonds [50].

The biohydrogenation of linoleic and linolenic acid involves a major pathway, but there are also many intermediates that can be produced through alternate pathways. This is one of the reasons why there are many different isomers of conjugated linoleic acid that are derived from alternate pathways of biohydrogenation. These alternate pathways can also contribute to CLA isomers that have also been implicated in the reduction of milk fat synthesis. **Figure 1.3** illustrates the alternate pathways that are involved in the biohydrogenation of linoleic acid.

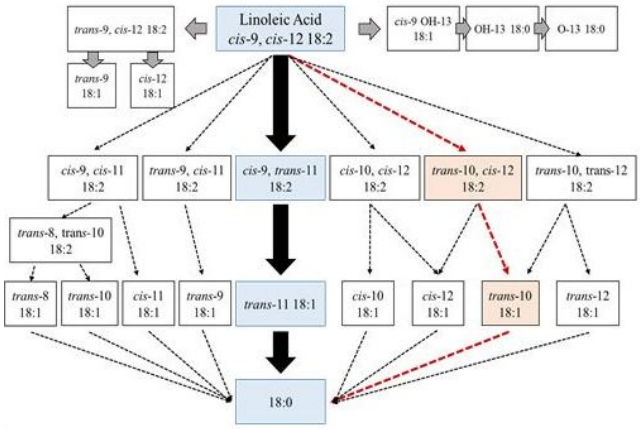

Figure 1.3: Major and alternate pathways of biohydrogenation and the subsequent production of CLA and 18:1 isomers. Dashed lines indicate minor pathways and solid lines highlight the major biohydrogenation pathway. Adapted from Chilliard et al. [51].

Microbial species involved in fatty acid biohydrogenation are well documented. Most notably, Harfoot et al. [52] described two classes of bacteria that are involved in biohydrogenation; group A and group B. Group A bacteria can isomerize and hydrogenate 18 carbon fatty acids to monounsaturated fatty acids (MUFA), specifically octadecenoic fatty acids. Group B bacteria are then able to hydrogenate and saturate the *cis* double bond to stearic acid. Under normal circumstances, group A bacteria isomerize linoleic acid primarily to *cis*-9, *trans*-11 CLA then hydrogenate to *cis*-9, *trans*-11 CLA to *trans*-11 C18:1. Group B bacteria then hydrogenates *trans*-11 C18:1 to stearic acid, thus fully saturating the original PUFA if allowed to go to completion.

It has been observed that apart from diets high in PUFA, high concentrate rations, particle size, and rumen unsaturated fatty acid load (RUFAL) can all contribute to milk fat depression due to their interactions with these groups of bacteria. Rumen unsaturated fatty acid load is a term used to describe the total unsaturated fatty acid supply entering the reticulo-rumen each day from feed and is calculated as the sum of oleic, linoleic, and linolenic acids.

It has been shown in several investigations that milk fat depression is most commonly induced due to excess PUFA, thus leading to a high RUFAL. Microbial populations within the rumen are quite sensitive to unsaturated fatty acids, and attempt to "detoxify" them by the process of biohydrogenation. Several studies have identified specific bacterial populations that are prevalent during conditions of milk fat depression.

Lantham et al. [53] reported an increase in the relative abundance of *Peptostreptococcus* and a decrease in *Butyvibrio spp.* during low-roughage induced milk fat depression. Thus, providing an early example of microbial changes that occur during MFD. More recently, *Megasphaera elsdenii*, a lactate utilizing bacterium, was reported to account for up to 4% of the 16S rRNA gene copy number of the total microbial community in cows experiencing MFD [54]. However, Weimer et al. [55] failed to induce MFD by dosing *M. elsdenii* that was isolated from the rumen. This result was not surprising in that the establishment of exogenous bacterial strains in the rumen is challenging, even if the bacteria was isolated from the donor cow. Maia et al. [48] observed that *Butyrivibrio fibrisolvens* produced primarily vaccenic acid in the presence of linoleic acid. The stearate producing bacterium *Clostridium proteoclasticum* did not grow in the presence of PUFA, indicating this population may be inhibited during MFD. Furthermore, Boeckaert et al. [56] noted that there was an increase in the *trans* C18:1 FA in the rumen that was related to changes populations of *Butyrivibrio spp*. Kepler et al. [57] demonstrated early on that populations of *Butyrivibrio fibrisolvens* were able to hydrogenate *trans*-10, *cis*-12 CLA to *trans* 10 C18:1, and therefore may play a major role in MFD. Other reports have shown that *Megasphaera elsdenii* and *Priopionibacterium* produce *trans*-10, *cis*-12 CLA and could also play a role in the onset of MFD [58,59].

Protozoa have been implicated to have an impact on biohydrogenation in the rumen as well. However, this has been debated for quite some time. Keeney [60] reported that protozoa make up approximately 75% of the total microbial FA contribution, and that they are an important source of PUFA, CLA, and *trans*-11 C18:1 [4,50]. Early observations noted an almost complete absence of rumen protozoa during period of MFD, and the logical assumption was that protozoa contribute to biohydrogenation [61]. Chalupa et al. [62] showed that holotrich protozoa, even at double the concentration normally found within the rumen, were unable to hydrogenate linoleic-1-14C acid. Later works revisited the protozoal role in biohydrogenation and concluded that protozoa do not directly synthesize CLA or *trans*-11 C18:1, but contribute to the slow of FA biohydrogenation by maintaining a ruminal pool of FA within their membranes that is unavailable to biohydrogenating bacteria [4,63].

Anaerobic fungi make up a smaller part of the (estimated at 8%) total microbial mass in the rumen, but produce a wide variety of fibrolytic enzymes that are essential for forage fermentation [52,64]. Therefore, it is logical to assume that they may play a role in biohydrogenation as well. Nam et al. [65] tested this hypothesis and found that ruminal fungi do hydrogenate linoleic acid, but at a much slower rate. It was also observed that *cis*-9, *trans*-11 CLA was an intermediate and *trans*-11 C18:1 was the end product, with *Orpinomyces* comprising the most active role of the fungal isolates tested. Due to fungi representing only a small portion of the microbial biomass, it can be concluded that biohydrogenation by rumen fungi do not substantially contribute to the overall biohydrogenation capacity of the rumen. However, it is important to note that bacteria are not the only microbial domain in the rumen that can hydrogenate certain FA.

Conjugated Linoleic Acid

Conjugated linoleic acids are natural components derived from rumen transformation of linoleic acid or desaturation in the tissues, and represent a group of positional and geometric isomers of linoleic acid (**Figure 1.2**; *cis*-9, *cis*-12 octadecadienoic acid). The term "conjugated" refers to a fatty acid with two double bonds separated by one single bond. It has been reported that there are more than 20 different types of known CLA produced in the rumen by its microbial communities, but MFD has only been induced consistently by three [66].

These CLA milk fat inhibitors (CLA_{MFI}) are absorbed and transported to the mammary gland, where essential enzymes for milk fat synthesis are inhibited, reducing the synthesis of milk fat. Milk contains moderate levels of CLA, and recent research has aimed to increase the CLA content in milk due to the anti-carcinogenic properties of the *cis*-9, *trans*-11 CLA isomer. Of the 20 different isomers of CLA, *cis*-9, *trans*-11 (rumenic acid) is considered to be the most common and abundant form of CLA. Approximately 17 of these CLA isomers have been identified in cattle ruminal contents [67].

In a study by Lee et al. [67], a stable isotope of linoleic acid was incubated in rumen contents for 48 hours, resulting in the formation of several CLA isomers, including *trans*-10, *cis*-12 CLA. This study was aimed at identifying CLA isomers that are derived from the biohydrogenation of linoleic acid.

In the normal biohydrogenation pathway, the *cis*-9, *cis*-12 double bond complex of linoleic acid is initially isomerized at the *cis*-12 position to form *cis*-9, *trans*-11 CLA **(Figure 1.4)**. The *cis*-9, *trans*-11 CLA is then converted to *trans*-11 C18:1 by the addition of a hydrogen atom. Conjugated linoleic acid in milk and meat of ruminants can be derived from two sources, either from ruminal biohydrogenation of linoleic acid or synthesis from *trans*-11 C18:1 by the animal's tissues.

The *trans*-11 C18:1 CLA can then be converted into *cis*-9, *trans*-11 CLA in the tissues by Δ-9 desaturase. Delta-9 desaturase activity is greatest in the mammary gland of lactating cows, and *cis*-9, *trans*-11 CLA can be endogenously synthesized at this level. When animals were abomasally infused with sterculic acid, milk fat content was reduced in lactating cows, thus indicating a relationship between sterculic acid and inhibition of Δ9 desaturase [34].

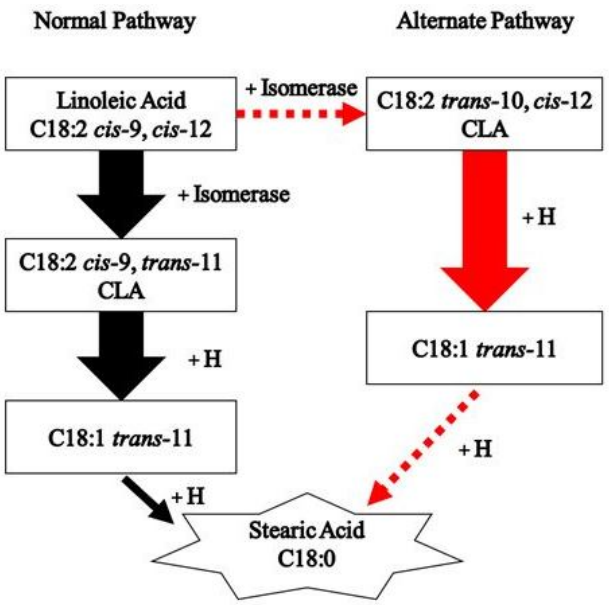

Figure 1.4: Biohydrogenation of linoleic acid in the rumen.

In the rumen, CLA is an intermediate of the linoleic acid to stearic acid biohydrogenation pathway. The previously outlined scenario is the normal pathway of biohydrogenation that these FA undergo. However, there is an alternate pathway that can produce *trans*-10 C18:1, and the potent milk fat inhibitor, *trans*-10, *cis*-12 CLA **(Figure 1.4)**. The normal pathway can shift to the alternate biohydrogenation pathway due to a variety of factors including excess RUFAL, high grain diets, or poor effective fiber, for example [68]. The *trans*-10, *cis*-12 isomer has been denoted as the most potent inhibitor of synthesis of milk fat [69]. In a study conducted by Baumgard et al. [69], abomasally infused *trans*-10, *cis*-12 CLA resulted in a curvilinear reduction in milk fat yield occurred with increasing amounts of the isomer. Other isomers have also been reported to depress milk fat yield. This is the case for *cis*-10, *trans*-12 CLA which was reported by Saebo et al. [70] to induce MFD. *Trans*-9, *cis*-11 CLA was also shown to increase when milk fat was reduced through chromatographic analysis of milk samples from milk fat depressed cows [71]. Perfield et al. [72] later confirmed this finding by using a CLA enrichment that supplied 5 g/d of *trans*-9, *cis*-11 CLA via abomasal infusion reduced milk fat yield by 15%.

Adding plant-derived oils to an animal's diet increases the milk fat concentration of CLA, but also may inhibit rumen microbial growth [34]. Conditions such as low forage, low fiber diets and/or increased intake of plant oils have decreased milk fat secretion in dairy cows. *Streptococcus* and *Lactobacillus* bacteria have been shown to produce *trans*-10, *cis*-12 CLA [48]. Changes in milk fat composition were usually paired with a reduction in fatty acids derived from Δ9 desaturase activity. Moreover, restriction of feed intake has been reported to increase milk fat CLA from mobilized body fat stores [73].

Milk Fat Depression

Protein and fat are two of the most valuable components in milk. The concentrations of these major components can be widely altered by a variety of factors including genetics, health status, and nutrition. Nutritional changes can affect the milk fat content positively or negatively, with high concentrate diets sometimes exerting negative effects. Milk fat depression was first reported by French chemist Jean Baptiste Boussingault in 1845, who observed that when beets were fed to dairy cows butterfat was reduced [34]. Boussingault attributed this phenomenon to the inherently low fat content of the diet. This idea was not unrealistic in theory, since under MFD conditions the mammary gland may not have sufficient FA supply for lipogenesis. However, ruminants cannot directly convert glucose to FA, and acetate and butyrate are utilized by the mammary gland for *de novo* synthesis and long-chain FA derived from the diet or adipose tissue mobilization, are secreted in the milk [17]. The specific cause of low milk fat syndrome continued to elude producers for many years and prompted investigations into strategic feeding practices in the last century to determine the cause of MFD [74-76]. In the early part of the 21st century, MFD was observed with a variety of diets including cod liver oil [77], plant oils [78], and diets with high concentrates [79]. Davis et al. [80] divided diets that cause MFD into two groups, the first comprising diets with large amounts of readily digestible carbohydrates and reduced fiber, whereas the second group includes diets with high amounts of PUFA. There are many diets that may fall into either category, but a common denominator in the onset of MFD revolves around management practices, other dietary components, or the physiological state of the animal. Therefore, the separation of diets that cause MFD into two groups does not adequately assess the risk for MFD.

Acetate and Butyrate Theory

Many theories have been postulated to explain the MFD phenomenon. One theory suggests that inadequate fermentation in the rumen results in a reduced amount of lipogenic precursors, such as acetate and butyrate, causing a subsequent reduction in milk fat synthesis [34]. It is logical to infer that changes in rumen VFA production may be responsible for a reduction in milk fat due to the role of acetate and butyrate in milk fat synthesis. This precursor-product-ratio theory was first introduced by Tyznik et al. [81] and is based on the premise that reduction of ruminal acetate and butyrate production can in turn inhibit synthesis of milk fat due to a substrate shortage for *de novo* FA synthesis. Changes in VFA patterns have been reported with MFD, but the changes are most closely associated with the forage to concentrate ratio (F:C) or with low amounts of effective fiber. Acetate has been shown to be markedly reduced in these scenarios, but this is mainly due to the increase in relative ruminal propionate production. Milk fat depression has been reported to occur in diets supplemented with oils without the presence of any changes in ruminal VFA concentrations [81]. This theory was disproved in a number of studies, where acetate was infused yet milk fat concentration remained unchanged. Van Soest [17] noted that historically the acetate deficiency theory was undoubtedly disproved by McClymont [82], who

found that glucose or propionate infusions produced low milk fat. However, acetate appears to alter milk fat in normal conditions when MFD is not present. In addition, Van Soest [17] expressed concern over the expression of VFAs as molar proportions without the total acid concentrations as well. Van Soest states that VFA proportions are of biological significance, but the value of the measurement is inherently skewed by the fact that changes in VFA concentration of one acid require a statistical change of the opposite sign in the other acids. In addition, it was due to this discrepancy that the "erroneous theory" that an acetate deficiency causes MFD. Further investigations into the exact cause of the onset of MFD continued after the abandonment of this theory.

Glucogenic-Insulin Theory

Mcclymont et al. [83] proposed a different theory, suggesting that high ruminal propionate concentrations and rates of hepatic gluconeogenesis can result in an increased circulating insulin spike, and thus, precursors available for milk fat synthesis might be limited due to an insulin-induced response. This theory gained traction due to the logical assumption that milk fat depression from high-concentrate feeding was due to increased ruminal propionate flux and subsequent stimulation of hepatic glucose synthesis. This would in turn increase the release of insulin. However, in the mammary gland of the ruminant, insulin is responsible for normal mammary cell function maintenance and is not affected by fluctuations in circulating meal-induced insulin spikes since it only requires relatively small quantities of insulin. The rate of lipogenesis and lipolysis in other ruminant tissues are affected by insulin, and may somewhat affect the nutrient availability to the mammary gland [34]. Furthermore, this glucogenic-insulin effect is thought to divert nutrients from the mammary gland due to the increase in circulating insulin levels, which result in the increase utilization of acetate, β-hydroxybutyrate, and dietary LCFA at the adipose tissue level. Therefore, there is a shortage of lipogenic precursors such as acetate and β-hydroxybutyrate for milk fat synthesis [34]. This theory has received considerable attention which prompted many investigations into its validity.

Davis et al. [80] summarized a number of trials that utilized infusions of propionate to test this theory, but reductions in milk fat yield were variable and the involvement of insulin in MFD remained unclear. Frobish et al. [84] infused glucose and propionate into the abomasum and no MFD was observed. The authors argued that the observations by McClymont et al. [83] were under physiological and that other feeding studies with propionate had shown MFD. Use of a hyperinsulinemic-euglycemic clamp has been tested in order to minimize disruptions in glucose homeostasis and hypoglycemia, but the effects on the synthesis of milk fat were minimal. The hyperinsulinemic-euglycemic clamp method works by raising and maintaining plasma insulin levels through a continuous infusion of insulin. When insulin levels hold steady, the glucose infusion rate equals glucose uptake by all the peripheral tissues. McGuire et al. [85] found that milk yield and milk fat yield remained unchanged during the insulin clamp study, yielding little support for the glucogenic-insulin theory.

Bauman et al. [86] noted that the changes in some of the insulin clamp studies were marginal, reporting milk fat yield reductions of less than 5% during some of these investigations. Corl et al. [87] reported a decrease in milk fat percentage and yield, but determined that this was a result of the ability of insulin to inhibit lipolysis and limit preformed fatty acids available to the mammary gland. Furthermore, insulin plays a major role in the regulation of lipolysis through metabolic triggers that mobilize fat stores or signal adipose to store FA and start lipogenesis based on insulin levels. However, in regards to the glucogenic-insulin theory it is believed that the differences in insulin clamp studies with respect to MFD were primarily due to energy balance [85,88-89]. This is further supported by Corl et al. [87], who determined that the observed reduction in milk fat could be attributed to the mobilization of body fat reserves due to the experimental cows being in very early lactation. Bauman et al. [66] reported that during a time of negative energy balance, MFD repartitions the energy available toward protein and milk synthesis. This critical period result in greater mobilization of FA to meet energy demands of the mammary gland as well as replenish body reserves, thus, the reduction in milk fat reported in these studies cannot be attributed to the glucogenic-insulin theory but rather energetic balance.

Vitamin B_{12}/Methylmalonate Theory

Similar to the previously discussed theory, Frobish et al. [84] presented the vitamin B_{12}/methylmalonate theory of MFD. Vitamin B_{12} is an essential component of the enzyme methylmalonyl CoA mutase, which plays a major role in propionate metabolism [86]. The concept is centered on reduced production of vitamin B_{12}, coupled with increased propionate, would cause an accumulation of methylmalonate within the liver that would then travel to the mammary gland and inhibit *de novo* FA synthesis through the down regulation of ACC and FAS. Often in cases where high concentrate diets are fed there is an observed increase in propionate and reduction in vitamin B_{12}. Walker et al. [90] reported that high concentrate diets could potentially limit the amount of vitamin B_{12} produced in the rumen, and later studies suggested that it could also be the cause of MFD in dairy cows fed low forage diets. However, Elliot et al. [91] could not confirm these findings and observed that vitamin B_{12} injections did not alter milk fat. This was later confirmed by Croom et al. [92], where vitamin B_{12} failed to correct the decrease in milk fat production associated with feeding low forage diets.

Trans Fatty Acid Theory

The *trans* fatty acid theory involved the idea that milk fat synthesis is depressed by *trans* octadecenoic acid that is produced through incomplete and modified rumen biohydrogenation. This theory forms the foundation of the most current and widely accepted biohydrogenation theory and was first suggested by Davis et al. [80]. Davis et al. [80] observed an increase in C18:1 and postulated that this response was mostly due to the accumulation of *trans* C18:1 during MFD. This theory was expanded upon by multiple studies that showed that *trans* C18:1 increased in the milk fat of a variety of diets related to

MFD [93-95]. *Trans*-11 C18:1 is an intermediate in the biohydrogenation pathway of linoleic acid **(Figure 1.3)** and linolenic acid. This FA is the most predominant *trans* octadecenoic acid isomer present in milk fat [33], so it is logical that an increase in this isomer during MFD may be the cause. However, investigations by Kalscheur et al. [96], and Selner et al. [97] demonstrated that there can be increased *trans* C18:1 due to dietary modification, and milk fat remains unchanged. Work by Griinari et al. [98] expanded upon this finding and reported that *trans*-10 C18:1 was to blame rather than all *trans* C18:1 isomers. *Trans*-10 C18:1 is also produced from the ruminal biohydrogenation of linoleic acid **(Figure 1.4)**, and is a major part of a minor pathway later proposed by Griinari et al. [99] that helped to formulate the most current and widely accepted theory. The *trans* fatty acid theory was centered around the concept of *trans* C18:1 isomers as the cause of MFD, but there were too many inconsistencies and the identification of specific isomers in later work confirmed that the theory had conceptual flaws. Further investigations into the *trans* FA theory indicate that *trans*-10 C18:1 does not directly control milk fat production, but may respond to dietary factors [100]. However, the concentration of *trans*-10 C18:1 has been consistently reported to negatively affect milk fat yield and acts more as a proxy for the identification of milk fat depression [47].

Biohydrogenation Theory

The discovery of an anti-mutagenic compound in fried ground beef in the 70s by Mike Pariza [101-103], which was later identified as conjugated linoleic acid, shifted attention to increasing the content of CLA in dairy products due to the potential health benefits. In an attempt to increase milk fat CLA, it was observed that infusion of a CLA supplement that included a mixture of CLA isomers (predominately *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA) severely reduced milk fat [104]. These investigations into enhancing milk CLA content gave a great deal of insight related to milk fat depression. After the discovery that CLA isomers inhibit milk fat production, investigations centered on determining which CLA isomer is responsible and identifying the proposed biohydrogenation pathway. Baumgard et al. [69] identified the potential culprit of MFD as the *trans*-10, *cis*-12 CLA isomer. Further work by Baumgard et al. [105] and Peterson et al. [106] showed that milk fat yield can be decreased by 40-50% with as little as 10 g/d of *trans*-10, *cis*-12 CLA. In addition, Baumgard et al. [105] characterized changes in milk fatty acid composition and reported a marked decrease in *de novo* fatty acids, a common observation seen with MFD. Griinari et al. [99] postulated the biohydrogenation theory in order to build upon the limitations of the trans FA theory and stated that under certain dietary conditions the pathways of rumen biohydrogenation change and produce fatty acid intermediates that can reduce milk fat synthesis. Work by Griinari et al. [98] confirmed that a dietary supply of PUFA and a change in microbial processes in the rumen were essential for diet-induced MFD. In addition, work by Loor et al. [107] demonstrated that exogenous supply of *trans*-10, *cis*-12 CLA decreased milk fat yield and composition, and that *trans*-10, *cis*-12 CLA reduces *de novo* FA synthesis and desaturation in the mammary gland.

Practical Implications of MFD

The majority of dairy herds have been able to reduce the incidence of MFD through sound nutritional management in their feeding program. However, even the best herd and nutrition program can be susceptible to MFD. Dietary ingredients available to dairy farmers are constantly changing depending upon price, availability, unexpected changes in nutrient composition, energy levels, and requirements to increase milk yield and components. Therefore, nutritional program manipulations can result in MFD within a short period of time. Dairy cows not exhibiting MFD syndrome also express CLA_{MFI}, but at concentrations too low to cause MFD. Once MFD has been established, the identification of cause and respective amelioration can take several months. Under current milk pricing system, MFD represents a major concern for dairy producers. Based on the February 2018 milk fat price of $5.20/kg (USDA-FMMO), each 0.1% units decrease in milk fat results in a loss of $0.19/cow/d (based on 36 kg milk/d; average US milk production/d). A 200 cow herd that decreased from 3.6% to 3.3% milk fat would lose $114/d or $1140/d on a 2000 cow farm. Therefore, mitigating milk fat depression is very important to increase dairy farm profitability.

Milk fat depression is a multifactorial metabolic disorder, with onset being attributed to a number of conditions including, particle size, RUFAL, and rumen pH. It has been suggested that if pH is increased, CLA_{MFI} will not be produced in such quantity as with low pH conditions. Jenkins et al. [108] observed an increase in pH coupled with a decrease in *trans*-10 18:1 and *trans*-10, *cis*-12 18:2 in continuous cultures supplemented with potassium carbonate. Although other factors may explain these effects, it is suggested that pH can improve rumen conditions and prevent the shift in CLA_{MFI} production. When ruminal pH is decreased, bacterial species whose activity is primarily in the second reductase step of the biohydrogenation pathway are reduced, resulting in an accumulation of these intermediates [108]. Fuentes et al. [100] demonstrated that when culture pH was lowered from 6.5 to 5.5 there was an observed shift in CLA production that resulted in an increase in CLA_{MFI}. However, Harvatine et al. [109] reported that MFD can appear even when pH is not reduced. This observation involves other contributing factors that can lead to the onset of MFD. Alterations of rumen microbial populations through dietary interventions to favor less accumulation of CLA_{MFI} have been investigated thoroughly. Replacing starch with sugar sources has been shown to reduce the risk for MFD without disrupting performance [8]. Rico et al. [110] observed that altering the fermentability of the diet through modifying dietary NDF resulted in recovery from milk fat depression. However, it remains unclear whether or not these conditions will continue to be favorable when dietary changes in starch degradability or sugar inclusion, for example, are modified with a high PUFA diet.

Cellular Signaling of Fatty Acids

The theory that CLA isomers were responsible for reductions in milk fat was first introduced by Bauman [66]. Conjugated linoleic acid was first recognized for its anti-carcinogenic

properties by Pariza in the late 70s and early 80s in fried hamburger [101-103]. However, the isomer associated with these effects, *cis*-9, *trans*-11 CLA, is one of many of bioactive isomers. Bioactivity refers to effects on downstream target genes in the mammary gland, and alterations in the microbial communities within the rumen. The isomer *trans*-10, *cis*-12, has been associated with milk fat depression, and it's believed that it suppresses genes responsible for milk fat production in the mammary gland, such as stearoyl-CoA desaturase 1 (SCD1), fatty acid synthase (FASN), acetyl-CoA carboxylase (ACACA), and glycerol-3-phosphate acyltransferase 6 (AGPT6) [111,112]. This *trans*-10, *cis*-12 CLA isomer was first recognized as a potent inhibitor of milk fat production [69]. It has been reported that this isomer decreases body fat in chickens and rabbits, but has not altered body fat stores in ruminants, suggesting that it has a greater capacity for targeted cellular control than previously thought [113-115]. Although the mechanism by which CLA affects body fat is unknown, it is suggested that it causes a negative energy balance by reducing energy intake, and increasing energy excretion through heat loss [115]. This may be similar to what happens at the mammary level. Studies in mice, pigs, and sheep where *trans*-10, *cis*-12 CLA was supplemented showed a coordinated decrease in milk fat content [116-118].

Nutrigenomics applies high-throughput genomic methods in nutrition research, and has become an integral part of understanding the interactions between nutrients and tissue metabolism. Small changes in gene expression are able to be measured by using techniques such as real-time PCR and microarray, with the latter being of great importance in studying the transcriptome [119]. Recent advances in nutrigenomics have allowed researchers to characterize nutrient-tissue interactions, and quite possibly begin to explain the underlying mechanism of MFD [68,120]. The *trans*-10, *cis*-12 CLA isomer has been reported as the most potent inhibitor of milk fat synthesis, but *trans*-9, *cis*-11 CLA and *cis*-10, *trans*-12 CLA isomers have also exhibited inhibitory effects on milk fat synthesis [70, 72]. Since fat is the only milk component affected by *trans*-10, *cis*-12 CLA, it appears that effects on the biochemical pathways involved in lipid synthesis in the mammary gland are highly specific [66].

Transcription factors play an intricate role in whether a particular gene is active, and interest in determining the "master regulators" involved in MFD have been investigated recently. Sterol regulatory element binding protein-1 (SREBP1; **Figure 1.4**) is highly active in mammary tissue. Although changes in gene networks associated with milk fat depression are not thoroughly understood, the activation of SREBP1 has been well described. In short, SREBP is associated with a chaperone protein known as SCAP, and forms a SREBP/SCAP complex within the ER, when inactive. Activation is accomplished through the dissociation of insulin-induced gene 1 or 2 (INSIG), which allows translocation to the Golgi. Once in the Golgi apparatus SREBP1 is proteolytically cleaved to form the active transcription fragment, nuclear SREBP1 (nSREBP1). This nSREBP1 transcription fragment is translocated to the nucleus where it can bind to sterol-regulatory elements and eventually stimulate transcription of genes that are involved in the regulation of lipogenesis [121]. Despite SCAP having integral parts in lipid regulation, some investigations have reported only small differences in SCAP expression or abundance with CLA induced MFD [122]. However, INSIG1 was reported to be reduced with *trans*-10, *cis*-12 CLA treatment by Vyas et al. [123] and Harvatine et al. [122]. Furthermore, INSIG1 has been implicated in the amount of *de novo* and preformed FA in the mammary gland [41]. The apparent sensitivity of INSIG1 and lack of an effect on SCAP indicate that the alterations in lipogenesis may be regulated by downstream gene networks associated with SREBP1. Work by Peterson et al. [124] reported a decreased abundance of SREBP1 during *trans*-10, *cis*-12 CLA inhibition of FA synthesis in MAC-T mammary epithelial cell cultures. Further, Harvatine et al. [122] observed a decrease in expression of SREBP1 in mammary tissue from cows experiencing MFD. Down regulation of SREBP1, SREBP1 activation proteins and SREBP1-regulated genes expression for key enzymes involved in fat synthesis in bovine mammary epithelial cells suggests a high influence of SREBP1 as a central signaling pathway in the regulation of FA synthesis. Recent work has shown that the isomer *trans*-10, *cis*-12 CLA inhibits fatty acid synthesis through down regulation of genes involved in *de novo* fatty acid synthesis in goat mammary epithelial cells, and this process is likely correlated to the activation of AMP-activated protein kinase signaling pathways [121]. Furthermore, milk fat production is energetically expensive, so it is logical to infer that there may be an energy sparing effect during MFD. Harvatine et al. [125] delivered 7.5 g/d of *trans*-10, *cis*-12 CLA over the course of 4 d and observed a substantial decrease in milk fat yield and content, despite milk yield and other components being unaffected. One of the key findings in this study is that during MFD, adipose tissue expression of adipogenesis enzymes including lipoprotein lipase, FASN, SCD, and FABP4 were all increased. In addition, key regulators of lipid synthesis, SREBP1, thyroid hormone spot 14 (S14), and PPARγ were also increased in adipose tissue. These findings suggest that during milk fat depression there is an energy sparing effect from the reduction in milk fat synthesis and thus shuttled toward adipose tissue fat stores during MFD.

Soluble Carbohydrates and Starch Degradability

Carbohydrates represent 60-70% of the DM in dairy cow rations compared to other dietary nutrients [126]. Nonstructural (NSC) and structural carbohydrates are the two classifications of carbohydrates, with the nonstructural fraction consisting of sugars, starches, organic acids, fructans, and structural carbohydrates being the fibrous components. Nonstructural carbohydrates are highly fermentable, and oftentimes used in diets requiring high amounts of dietary energy, such as a lactating dairy cow ration. Nonfibrous carbohydrate (NFC) is a term that cannot be used in place of NSC, since pectin is included in NFC, but not in the NSC fraction. Organic acids make up a large difference in the NSC and NFC. Diet fermentability plays a major role in many of the nutrient interactions within the rumen. Highly fermentable diets have been implicated in the induction of dietary syndromes such as acidosis and milk fat depression. Work by Rico et al. [110] showed that milk fat synthesis increased significantly when diet fermentability and PUFA content was decreased. Other strategies have included the

use of rumen modifiers to improve milk fat production. The methionine analog, 2-hydroxy-4-(methylthio)butanoate (HMTBa), has been shown to increase milk fat when feeding highly fermentable diets [127,128]. Baldin et al. [129] investigated the effect of diet fermentability and HMTBa supplementation and observed that cows with a high risk of biohydrogenation–induced MFD had greater milk fat with HMTBa. It was suggested that the HMTBa decreased the absorption of alternate biohydrogenation intermediates that are responsible for the onset of MFD [129]. As MFD is the result of the interaction of numerous factors, multiple dietary components have to be evaluated, as they have the potential to shift rumen microbial populations and alter the production of biohydrogenation intermediates.

Neutral Detergent Soluble Fiber

Sources high in starch, such as corn, or other grains, are common feedstuffs, and represent a large portion of energy that can be made available to the cow. However, starch can be quickly degraded and lead to acid accumulation and low rumen pH. Low rumen pH contributes to subacute ruminal acidosis (SARA), and can also result in shifts in the biohydrogenation pathway associated with milk fat depression. Therefore, replacing starch with other fermentable carbohydrate sources is of interest. The neutral detergent-soluble carbohydrate (NDSC) fraction is defined as a rapidly fermentable energy source for ruminal microbial growth and energy available for use by the cow. The NDSC fraction includes organic acids, monosaccharides, oligosaccharides, starch, fructans, pectin, β-glucans, and are not recovered in the NDF fraction [130]. Pectin and β-glucans are structural carbohydrates, but are soluble in neutral detergent solution, and are therefore classified as neutral detergent-soluble fiber (NDSF). Pectin is the predominant carbohydrate in NDSF, and possesses different fermentation and digestion than its NDSC fraction counterparts. Fermentation of pectin generates more acetate, less propionate, and lactic acid, with a greater rate of digestion when compared to NDF, but slower digestion than sugar or starch [131]. Sources high in pectin include legume forages, soybean hulls, beet pulp, and citrus pulp [132,133]. In some reports, replacing corn with non-forage fibrous byproducts increased total tract starch digestibility [134]. Even though NDSF is also fermented rapidly in the rumen, it is not fermented to lactate and will not continue fermenting when pH reaches a nadir [135]. Additionally, the use of sources rich in pectin have been shown to increase microbial protein synthesis, suggesting that NDSF can provide similar sources of energy compared with starch for supporting essential ruminal microbial growth [136]. Feedstuffs high in NDSF may help to improve animal production and the ruminal environment when challenged with a diet high in PUFA **(Table 1)**. However, this has not been extensively investigated. In a recent investigation by Santos-Silva et al. [137] cereal grains were replaced with dried citrus pulp in high soybean oil diets fed to ewes it was observed that milk fat yield was unchanged, but milk yield was increased with citrus pulp. In a similar study conducted using lactating Holstein cows, Santos et al. [138] found that milk fat yield or composition showed no improvement with the addition of citrus pulp. However, both of the aforementioned studies aimed at investigating the effect on the milk or milk solids. Assis et al. [139] observed that a total replacement of corn with citrus pulp in lactating cows will not alter production, and Santos et al. [138] reported an increase in milk production where citrus pulp replaced corn up to 14% of the diet DM. In a review by Bradford et al. [140], it was noted that high-producing dairy cows may benefit from dietary inclusion of nonforage fiber sources. *Butyrivibrio fibrisolvens* has been reported to play a major role in the conversion of linoleic acid to CLA and then hydrogenation to trans-11 C18:1. This bacterium has starch and pectin degrading abilities, which may have major implications for diets high in NDSF and PUFA [141]. Moreover, Solomon et al. [141] observed that a high pectin diet with full fat extruded soybeans did not reduce milk fat. Thus, it is of interest to evaluate the effect of replacing a portion of starch with ingredients rich in NDSF.

Table 1: References that have investigated soluble carbohydrates and their effect on milk fat composition and yield, milk yield, rumen pH, biohydrogenation, CLA isomer production. K_d=starch degradability.[1]

Soluble Carbohydrate			
Item	**Soluble Fiber**	**Sugars**	**Starch Kd**
Milk Fat Yield	$+^{bde}$, $-^{f}$	$+^{gk}$	No effect^{lmn}
Milk Fat %	$+^{bde}$, $-^{f}$	$+^{gk}$	No effect^{ln}, $-^{ms}$
Milk Yield	No effect^{bc}	No effect^{g}, $-^{k}$	$+^{m}$
Biohydrogenation	$+^{r}$	$+^{jk}$	$-^{lo}$
pH	$+^{aq}$, $-^{p}$	No effect^{g}, $+^{hk}$, $-^{i}$	$-^{l}$, $+^{o}$

[1]a=Strobel et al. [131]; b=Mansfield et al. [142]; c=Voelker et al. [9]; d=Van Knegsel et al. [143]; e=Shahmoradi et al. [144]; f=Boguhn et al. [145]; g=Broderick et al. [6]; h=Heldt et al. [146]; i=Kellog [147]; j=Ribiero et al. [148]; k=Martel et al. [4]; l=Harvatine et al. [149]; m=Theurer et al. [150]; n=Dann et al. [151]; o=Lascano et al. [13]; p=Naderi et al. [5]; q=Leiva et al. [132]; r=Santos-Silva et al. [137]; s=Bradford et al. [25].

Sugars

Sugars such as sucrose and feeding molasses have been investigated in their influences on ruminal pH, VFA, biohydrogenation, and milk fat production **(Table 1)** [4,6,152]. Sugars can provide another source of energy in the rations of lactating dairy animals that provide a rapidly degraded source of carbohydrates. There has been recent interest in using sugar to replace a portion of the starch due to the increasing demand and price of corn and starch sources [140]. Sugars can be beneficial in that they require little enzymatic activity to cleave the disaccharide and provide an energy source for the rumen microbes. It has been proposed that utilization of sugars in dairy rations could help prevent drops in pH and promote fiber digestibility [7,153,154]. This positive influence could minimize acidosis problems and limit the risk of milk fat depression. Martel et al. [4] reported that addition of 5% molasses in the diet can improve milk fat synthesis, increase ruminal pH, and increase ruminal biohydrogenation. The increase in ruminal pH is of importance due to the effects CLA_{MFI} that are prevalent in periods of low ruminal pH. However, Oba [155] reported that a large majority of *in vivo* studies found no effect of sugar on pH, but that the few studies that do report increases in pH replaced a portion of the starch with sugar. The theory behind how addition of molasses can alter ruminal pH is suggested to be due to the production of butyrate. Molasses increases butyrate production, thus, stimulating blood flow and potentially increasing uptake rate of volatile fatty acids across the rumen epithelium. Guan et al. [156] observed that greater butyrate concentration in rumen fluid was associated with greater feed efficiency of beef cattle. Some *in vitro* studies have shown that sugar fermentation results in increased butyrate [157,148], but several *in vivo* studies were unable to replicate this finding [155]. Oba et al. [158] attributed this discrepancy due to the rapid fermentation of sugar immediately after consumption and because butyrate is absorbed faster than acetate or propionate and therefore changes may not be able to be detected. Furthermore, sucrose addition had greater molar proportions of butyrate and higher ruminal pH as compared to lactose [158]. The effects of feeding sugar and subsequent enhanced butyrate absorption on gut proliferation and overall nutrient metabolism in ruminants warrant further investigation. However, Oba et al. [158] also found an increase in the relative mRNA abundance of genes responsible for the facilitation of absorption and fermentation of ruminal VFA with dosing of sucrose or lactose. This observation sheds light on the increases in rumen pH due to enhanced ruminal epithelial permeability of VFA which has been observed despite the rapid fermentation rates of sugars. The responses seen with sucrose but not lactose are most likely due to the more rapid fermentation of sucrose. Sucrose is a disaccharide of glucose and fructose, while lactose is composed of glucose and galactose. The two disaccharides are similar in that each consists of one glucose monosaccharide, but they differ only by fructose and galactose. Weisbjerg et al. [159] reported that sucrose hydrolyzes at a much faster rate than lactose, and Sutton [160] noted that fructose and glucose ferment faster than galactose. Weisbjerg et al. [159] found that sucrose had a hydrolysis rate of 1200-1404%/h^{-1} and was not increased by addition to the basal diet, whereas lactose had a much lower rate of hydrolysis (248%/h^{-1}) that was increased to 540%/h^{-1} when added to the basal diet. Some investigations have reported fructose increases the molar proportion of ruminal propionate, while lactose reduced propionate [161]. Propionate, a glucogenic precursor, can stimulate the release of insulin. There is some evidence that after sucrose administration propionate concentration is increased in the rumen and a concordant increase in plasma insulin when compared to starch or lactose dosing [162]. Furthermore, lactose has also been shown to increase rumen butyrate concentrations in some reports, with the increase potentially also causing some changes in ruminal epithelial tissue that results in greater VFA absorption [163]. This is logical in that rumen pH influences the rates of VFA absorption passive diffusion and protein mediated transport [17]. Chibisa et al. [163] found that lactose supplementation led to an increase in Cl^- competitive absorption of acetate and propionate, which suggested that carrier-mediated transport of dissociated acetate and propionate was upregulated. The authors noted that this could have potentially increased HCO_3^- influx into the rumen and explains why they did not see a drastic drop in rumen pH with lactose administration.

Many investigations have aimed at replacing a portion of the starch with sugar and reported positive responses with pH, DMI, milk production, and milk fat yield [155,163]. Supplementing sugar sources high in sucrose, such as molasses, to lactating cows has been reported to result in increased DMI [6,7,152]. However, these reports in DMI response to sugar supplementation have been noted to vary due to the importance of nutrient synchronization with nitrogen, forage inclusion in the diet, and forage quality [152]. Sun et al. [164] found that replacing dietary starch with sucrose inhibited the *trans*-10 biohydrogenation pathway and may account for the positive effects seen in milk fat production in subsequent investigations. The effects of feeding high-sugar diets on milk fat production and energy metabolism have been consistently reported in the literature [155]. Several studies have reported that cows fed diets high in sugar increased [6,7] or tended to increase [165] milk fat yield. These responses may partly be attributed to reduced *trans*-fatty acid production in the rumen. Incomplete biohydrogenation of unsaturated fatty acids is a primary cause of milk fat depression [166], but Ribeiro et al. [148] demonstrated that biohydrogenation of unsaturated fatty acids decreased linearly with sucrose addition in continuous culture media. In agreement with their findings, Penner et al. [7] showed that feeding a high-sugar diet decreased C18:1-*trans* fatty acid concentration in milk fat, and tended to increase milk fat yield. There has been a recent interest in the addition of sugar to rations as a means to replace a proportion of the starch. This dietary modification can potentially help control rumen pH, particularly if part of the starch is replaced while maintaining constant NFC. In a recent study by Razzaghi et al. [167], sucrose was supplemented in combination with sunflower seed, and an increase in rumen pH was observed along with a tendency for a decrease in the concentration of total *trans* 18:1 FA. The interactions between fatty acids, starch, soluble fiber, and sugar need to be investigated in order to determine how they influence rumen fermentation, biohydrogenation, and animal production.

Amounts of NSC and NFC to be fed are not well outlined, with the concentrations depending on the interrelationships of starch on ruminal fiber digestion, site of starch digestion, dry mater intake (DMI), replacement of NDF with NSC or NFC, and the method of processing. The NRC [126] suggests the NSC concentration in a diet should not exceed 30-40 percent of ration (DM basis) and NFC 2 to 3% higher, with the latter difference being due to the inclusion of pectin within the NFC fraction. The amount of dietary NFC has an effect on fermentation parameters and ultimately milk fat percentage.

Starch Degradability

Starch accounts from 50 to 100 percent of the NSC in most feeds, and the rate of ruminal digestion is paramount in determining the amount of starch that can be added to a diet in safe quantities [126]. Processing plays an important role in starch degradability, with particle size reduction and heat processing being methods of altering the feedstuff and increasing or decreasing starch availability [126]. The interrelationships between nutrient fractions, such as starch, may play a pivotal role in the biohydrogenation of these unsaturated fatty acids and ultimately affect the quantity and type of fatty acids that escape to the duodenum. Ruminal microbes require a supply of fermentable carbohydrates for growth and protein synthesis. Starch provides a substrate for this microbial growth and greater ruminal degradability [168]. Increasing the degradability of starch in cereal grain sources has been investigated using various processing methods such as dry rolling and steam flaking [169]. Differing processing methods vary in degradability and fermentability. However, increased starch content and degradability may lead to low ruminal pH conditions, which affect biohydrogenation, animal performance, fiber digestibility, and microbial populations [170,171]. Hatew et al. [172] reported differing levels of starch degradability *in situ* can effect methane production. Increased starch degradability resulted in reduced enteric methane production, suggesting that fermentation of starch favors production of propionate, creating an alternative hydrogen sink for methanogens.

A study by Bradford et al. [25] characterized production responses to low forage diets that included dry ground corn or high moisture corn. The high moisture corn depressed milk fat content, but the authors recognized that cows producing over 45 kg/d of milk had a higher milk fat content with the high moisture corn diet than low producing cows. It was suggested that high producing cows have a greater capacity for biohydrogenation of FA and decreased delivery of milk fat inhibiting isomers to the mammary gland. This observation is interesting but it should be noted that the degree of processing and incorporation of the corn sources can vary, as well as production responses. There is extensive literature on processing methods for corn and small grains and the subsequent effects on milk production, animal performance, and digestibility of nutrients [150]. Ensiling of high moisture corn or steam treatment of dry corn, for example, breaks down the hydrophobic starch-protein matrix and increases starch digestibility [134]. Corn has one of the greatest starch densities of the cereal grains commonly fed to livestock, and as grain processing becomes more extensive the starch within is generally made more available for digestion [150]. Gelatinization is a process that is typically involved in the application of heat and moisture to the kernel in the amorphous region and later moving to the crystalline regions. Grinding corn when compared to dry rolling corn increases the mean particle size, thus, increasing starch digestibility and NE_L by increasing the available surface area for bacteria to adhere to as well as opportunities for enzymatic degradation [11,173]. Steam flaking of corn or sorghum was reported to increase total milk yield and protein percentage, but decrease milk fat percentage [150]. Utilization of feedstuffs such as steam flaked or high moisture corn can help to improve flexibility and efficiency of dairy production, but should be carefully evaluated to ensure minimization of acidosis, depressed fiber digestibility, and potential reductions in milk fat production.

Ruminal Fatty Acid Metabolism using Continuous Culture Systems

Continuous culture systems have been utilized for many years and improved upon to address major limitations. Some of the major reasons why the improvement upon these systems has been of interest to ruminant nutritionists is that continuous culture systems are relatively inexpensive to operate and provide a cheaper alternative to test preliminary hypotheses when compared to running an *in vivo* trial. However, some discrepancies still exist between the accuracy of continuous culture systems and *in vivo* trials. Prior to the adoption of continuous culture systems batch culture was utilized, but a major pitfall of the batch culture system is they are unable to remove fermentation end products as well as be operated in a stable condition for long periods of time.

Compared to *in vivo* trials, continuous culture systems are often unable to maintain protozoal populations, although some designs have been modified to improve this [174]. There have also been reports of continuous cultures having major differences in dilution and passage rates, feed input, and lack of the ability to account for absorption parameters [175]. Mansfield et al. [176] investigated the fermentation and microbial ecology of *in vivo* and *in vitro* systems and found that concentrations of bacteria were greater, cellulolytic bacteria were also greater, and protozoal populations were greater *in vivo* when compared to *in vitro*. Warner [177] was able to maintain a microbial population *in vitro* for 4 d *via* the use of a dialysis sac with a buffered solution. However, the microbial population declined rather rapidly due to the accumulation of fermentation end products. Davey et al. [178] observed consistent bacterial numbers *in vitro* that compared to a 2 week animal experiment comparison. Furthermore, Slyter et al. [179] reported similar fermentation shifts *in vivo* and *in vitro* and found that protozoal concentrations decreased from 105 per ml to a level of 2 x 103 per ml after 4 d. In a meta-analysis by Hristov et al. [175] it was found that continuous culture systems typically have lower total VFA and acetate concentration, low or no protozoal populations, and lower OM and NDF digestibility. However, despite these differences continuous culture systems provide an advantage for the quick and safe assessment of experimental treatments. In addition, the utilization of

continuous culture systems for studies requiring the analysis of fatty acids in the culture and the effluent provide a great advantage over *in vivo* systems. Rumen digesta samples are typically simple to obtain and provide a picture of FA metabolism within the rumen. However, FA profiles of rumen fluid may not provide adequate information as to what is available for absorption post-ruminally. Omasal sampling allows researchers to evaluate FA concentration flowing out of the rumen, and thus, available to the animal [179]. However, this process can be difficult and labor intensive. Continuous culture systems possess a reaction vessel that acts as the rumen, and the overflow port where the effluent is removed would be comparative to the omasum in a cow. Sampling of overflow and analyzing for FA composition provide a more accurate representation of what is escaping the rumen and potential milk fat inhibiting isomers that would be available at the intestinal level in the animal. Furthermore, these *in vitro* systems incubate ground feed with buffer-diluted rumen fluid and are not expected to maintain viability of all microbial populations. Baldin et al. [180] stated that, the microbial populations involved in BH have not been fully characterized, and it is not clear if all key populations are culturable, even in mixed cultures. This is an important consideration in the field of milk fat depression and emphasizes that there is still much more investigation that is warranted.

Amelioration of Milk Fat Depression

Milk fat is one of the most energetically expensive and variable components in milk that is directly altered by nutrition and physiological state [181]. Some nutritional factors that can affect milk fat production include the amount of fiber in the ration, use of ionophores, feeding frequency or pattern, and type of fatty acids in the feed. These factors, combined with other non-nutritional factors such as genetics, stage of lactation, or parity, influence how much milk fat will be produced by the mammary gland. Three theories involved in MFD are the dietary fat deficiency, acetate deficiency, and the glucogenic-insulin theory. However, overall studies provide very little evidence for these theories involving a shortage of precursors for milk fat synthesis as the mechanism of diet-induced MFD. Further, MFD has been shown to occur when acetate is unchanged, thus, disproving the acetate deficiency theory [80]. It is widely accepted that there are a multitude of factors that can contribute to the induction of MFD. McCarthy et al. [182] reported that milk fat *trans*-10 C18:1 had a negative relationship to herd milk fat percentage as did small TMR particle size. However, the authors were unable to find some univariate relationships with herd-level milk fat. This further suggests the idea that many factors contribute to milk fat content, and herds experiencing low milk fat will need to multiple potential risk factors to solve the problem.

Milk fat depression occurs concordantly with alterations in rumen fermentation as well as the dietary inclusion of PUFA. There are several approaches in order to modify these interactions including diet fermentability, total RUFAL, effective fiber, or the use of rumen modifiers, for example. An example of early work on dietary modification to ameliorate MFD is that of Chalupa et al. [76]. The authors tested if the small amounts of forages in the form of corn silage or baled hay can correct milk fat depression when pellets are provided as the sole source of forage. Not surprisingly, animals receiving pellets continued to have depressed milk fat, but those receiving supplemental forages experienced an increase in milk fat and an increase in *de novo* FA of milk. The particle size of the diet was thought to be a primary cause for MFD at one point in time, however, we know now that it is more of a factor that contributes to the onset. In addition, work by O'Dell et al. [183] concluded that grind size plays an important role in MFD, and that a grind size of 0.64 cm induces MFD. Grant et al. [184,185] looked at feeding TMR's with different grind sizes of alfalfa hay or silage and observed a decrease in milk fat for the animals receiving the finely ground treatments. The reasoning behind why milk fat dropped was associated with increased passage rate and decreased digestibility, as well as alterations in VFA [186]. O'Dell et al. [186] reported that increasing feeding frequency to 4 times daily will prevent milk fat depression, but will not recover cows that are already experiencing milk fat depression. Another attempt at recovery was made by Emery et al. [187], who investigated the addition of sodium and potassium bicarbonate addition to cows receiving high-grain rations and found that rumen pH was increased and the bicarbonates prevented a decline in milk fat. Such early works paved the way for common practices that are in place on many dairy farms today and are examples of good management practices overall for a milking herd. After the discovery of the *trans*-10, *cis*-12 CLA and confirmation of the biohydrogenation theory, many works focused on identifying other isomers that may reduce milk fat or investigating the mechanism rather than trying to identify other dietary modifications that can be made to ameliorate MFD [72,188,189].

Recent studies have helped to identify the time course of induction and recovery from MFD, and if certain dietary modifications can fix it [2]. Rico et al. [2] were the first to investigate the time course of induction and recovery and found that MFD was induced by 3-5 d, and recovery achieved at 15-19 d. Further work by Rico and others aimed at investigating recovery from milk fat depression by utilizing a variety of techniques [110,190]. In a study by Rico et al. [110] MFD occurs within 7-10 days following a dietary change and 10-14 days is expected to recover milk fat to normal levels after dietary corrections. Methods utilized during recovery phases included modifying diet fermentability by increasing NDF decreasing PUFA concentration. In addition, the authors investigated the changes in ruminal microbial populations during the induction and recovery from MFD and observed that fungi and ciliate protozoa decreased by more than 90% during the induction phase and increased during the recovery phase. Although protozoa do not contribute directly to the biohydrogenation of linoleic acid, they play a major role in starch sequestration and may alter the rumen environment by engulfing large amounts of fermentable starch [191]. *Megasphera elsdenii* was increased during induction of MFD in the latter study, and has been associated with the production of *trans*-10, *cis*-12 CLA [48,191]. Populations of *Butyrivibrio* have been associated with fatty acid biohydrogenation, and can be inhibited by large amounts of

PUFA in the diet [48]. Because rumen microbial populations have been reported to change during MFD, there was also some early work that addressed this with respect to MFD. Satter et al. [192] switched from a high or low forage diet and tested the effect of diet with or without an exchange of rumen contents from cows fed the same diet. They observed that both induction of and recovery from MFD were not only aided by dietary manipulation, but also rumen inoculation from the donor cow. This paved the way for further work by Rico et al. [190], who utilized a similar approach and found that milk fat yield was not altered, but that *de novo* FA in the milk and rumen FA biohydrogenation was accelerated with inoculation.

Ionophores are commonly fed in dairy cattle diets due to their ability to shift the rumen microbial population to favor more gram-negative bacteria and are important when discussing MFD. Positive effects associated with ionophores include a shift in the acetate:propionate ratio towards more propionate, increased milk production, and reduced risk of acidosis by inhibition of lactic acid production [193]. Current commercially available ionophores include monensin (Rumensin®, lasalocid (Bovatec®), and laidlomycin propionate (Cattlyst®). Their mode of action involves the disruption of ion transfer of Ca^{2+}, Na^{+}, H^{+}, and K^{+}, and may interfere MFD amelioration. However, ionophores may inhibit linoleic acid biohydrogenation by altering the microbial populations responsible for a majority of lipid modification [194]. Several reports have characterized the effects of ionophores, such as monensin, on milk fat yield and concentration [193,195]. There has been some evidence that monensin reduces milk fat yield [195] and others that observed no change [196]. It is evident that the effects of monensin on biohydrogenation and milk fat production are dependent on the nature of the diet. Rico et al. [194] investigated if monensin has an effect on recovery from milk fat depression and found that monensin altered biohydrogenation and milk fat production minimally, but preformed FA in the milk were reduced with monensin. The variable responses with the use of ionophores in diets with high PUFA content suggest that it may be advantageous to exclude them when investigating milk fat depression and recovery.

Despite the often negative connotation of MFD on farm profits, there are instances where controlled MFD may be advantageous. For example, Baumgard [181] noted that milk fat is the most energetically expensive milk component to synthesize and could improve cow health in periods of heat stress or during early lactation. A better understanding of the ruminal transformation and tissue metabolism of fatty acids that potentially alter lipid production provides opportunity to control milk fat at energetically costly periods or physiological stress.

Since MFD is a multifactorial disorder, it is of great importance that we gain a deeper understanding of the nutritional effects on the metabolism of these bioactive fatty acids within the rumen. In some instances, utilization of different nutritional strategies, such as altering the soluble carbohydrate fractions or starch degradability, may help to improve ruminal fermentation and biohydrogenation of linoleic acid. Interactions between nutrient synchronization in the rumen with the use of sugars or neutral detergent soluble fiber with high oil diets are of interest to investigate if MFD can be improved with the addition or subtraction of certain nutrients. **Figure 1.5** illustrates some of the many factors involved in milk fat depression and their link to one another. The complexity of the rumen, tissue metabolism, and other factors all contribute to the onset of this disorder.

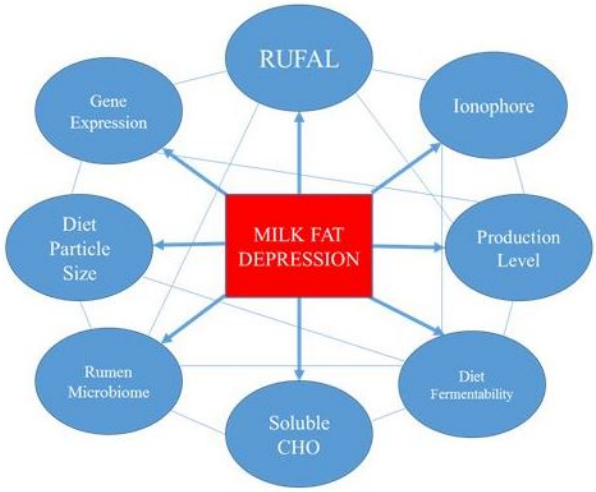

Figure 1.5: Factors linked with milk fat depression and their interrelationships.

Conclusion

The complexity of milk fat depression represents a specific challenge for producers. Feeding dairy cows to meet their nutritional requirements is challenged by the needs to meet energy demands without reducing milk and milk fat yield. Factors such as carbohydrate synchronization and microbial protein synthesis, impact on certain microbial populations, and the rumen pH can alter the biohydrogenation of fatty acids and their influence post-rumen. Starch degradability plays an integral role in rumen carbohydrate metabolism and recovery from milk fat depression due to the rate in which starch is fermented can influence microbial populations, rumen pH, and production of milk fat inhibiting isomers. Sugar has shown to be a promising strategy to enhance biohydrogenation and reduce the accumulation of milk fat inhibitors. Modifying other carbohydrate fractions, such as the soluble fiber fraction, is another potential approach to improving conditions within the rumen associated with diet-induced milk fat depression. However, studies investigating nutrient interactions and the effect on omasal outflow are cost-prohibitive *in vivo* and continuous culture systems provide a similar estimate of what would occur in the animal.

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