



## Invited review: Role of rumen biohydrogenation intermediates and rumen microbes in diet-induced milk fat depression: An update

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### ABSTRACT

To meet the energy requirements of high-yielding dairy cows, grains and fats have increasingly been incorporated in ruminant diets. Moreover, lipid supplements have been included in ruminant diets under experimental or practical conditions to increase the concentrations of bioactive n-3 fatty acids and conjugated linoleic acids in milk and meat. Nevertheless, those feeding practices have dramatically increased the incidence of milk fat depression in dairy cattle. Although induction of milk fat depression may be a management tool, most often, diet-induced milk fat depression is unintended and associated with a direct economic loss. In this review, we give an update on the role of fatty acids, particularly originating from rumen biohydrogenation, as well as of rumen microbes in diet-induced milk fat depression. Although this syndrome seems to be multi-etiological, the best-known causal factor remains the shift in rumen biohydrogenation pathway from the formation of mainly *trans*-11 intermediates toward greater accumulation of *trans*-10 intermediates, referred to as the *trans*-11 to *trans*-10 shift. The microbial etiology of this *trans*-11 to *trans*-10 shift is not well understood yet and it seems that unraveling the microbial mechanisms of diet-induced milk fat depression is challenging. Potential strategies to avoid diet-induced milk fat depression are supplementation with rumen stabilizers, selection toward more tolerant animals, tailored management of cows at risk, selection toward more efficient fiber-digesting cows, or feeding less concentrates and grains.

**Key words:** biohydrogenating bacteria, biohydrogenation theory, mammary lipogenesis, ruminant, *trans*-10 shift

### INTRODUCTION

Farm animals have been undergoing human-managed selection since their original domestication. In the last 60 yr, breeding programs have focused on the genetic improvement of production traits, such as milk yield of dairy cows (Oltenacu and Broom, 2010). To meet the energy requirements of those high-yielding dairy cows, grains and fats have often been incorporated in ruminant diets (Plaizier et al., 2008; Palmquist and Jenkins, 2017). Moreover, lipid supplements have been included in ruminant diets under experimental, as well as practical conditions, to increase the concentrations of bioactive n-3 fatty acids (FA) and CLA in milk and meat (Ganesan et al., 2014) and enhance cows' reproductive performance. However, such feeding practices might increase the incidence of diet-induced milk fat depression (MFD) in dairy cattle (Bauman and Griinari, 2003) and, less frequently, in small ruminants (Carreño et al., 2016; Fougère et al., 2018).

In the following sections, we will provide background of diet-induced MFD, beginning with its definition, and give an update on the role of rumen biohydrogenation intermediates in MFD since the review of Shingfield and Griinari (2007), based on recent studies in which associations were investigated between rumen or milk FA and decreases in milk fat. Furthermore, this review also provides a summary of the role of rumen microbes in MFD, based on pure culture studies and recent insights provided by molecular techniques. Finally, potential strategies to reduce the risk of MFD are discussed. Although this review particularly focuses on diet-induced MFD in dairy cows, differences and similarities between the response of cattle and small ruminants to diets associated with MFD will also be discussed.

Data used in the linear regression analysis of this review were derived from publications found via Google Scholar using the key words "milk fat depression," "milk fatty acid," and "rumen fermentation," published between 2008 and 2018. To investigate the effects of specific traits (i.e., rumen pH, rumen proportion of

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acetate or propionate, milk fat proportion of *trans*-10:*cis*-12 CLA or *trans*-10 18:1) on milk fat content or yield, a linear regression analysis was done using the MIXED procedure of SAS (version Enterprise Guide 7.1; SAS Institute Inc., Cary, NC) with the respective traits as independent variables (fixed effects) and study as a random effect (St-Pierre, 2001). When the data suggested an exponential curve, a natural log-transformation was performed before linear regression analysis.

### WHAT IS MFD?

Diet-induced MFD in dairy cattle is classically characterized by a reduction in milk fat content and yield with concomitant changes in ruminal biohydrogenation pathways, with no change in milk yield or in the yield of other milk components (Bauman and Griinari, 2001). Accordingly, this definition excludes situations with impaired milk fat yield resulting from lower milk yield, such as that due to decreased energy intake. However, there is some diversity in diet-induced MFD phenotypes, which implies that some MFD conditions do not neatly fit in this classical definition. Indeed, in some studies with fish oil supplementation (Ahnadi et al., 2002; Kairenius et al., 2015), decreases in milk fat concentrations were also accompanied by reduced milk fat synthesis and lower total milk yield. An increase in milk yield has even been described in ewes receiving fish oil, but associated downregulation of key genes involved in the mammary lipogenesis process confirmed nutrigenomic mechanisms rather than a milk dilution factor being responsible for the reduction in milk fat concentration (Suárez-Vega et al., 2017). Whether they fit in the classical definition of MFD or not, the common feature of diet-induced MFD conditions is the alteration in ruminal biohydrogenation pathways, which usually includes the well-known *trans*-11 to *trans*-10 shift (Alves and Bessa, 2014; Zened et al., 2016; Dewanckele et al., 2019b). These alterations in ruminal lipid metabolism are accompanied by changes in milk FA toward a reduced proportion and yield of short- and medium-chain FA, whereas proportions of longer-chain FA increase, although their yield usually remains constant or is decreased (Lock et al., 2008; Rico and Harvatine, 2013; Toral et al., 2015). This suggests a more pronounced inhibition of de novo synthesis than preformed FA uptake in the mammary gland (Bauman and Griinari, 2001; Bauman et al., 2011). The few situations in which preformed FA uptake seemed to be more strongly affected than de novo FA synthesis (on a molar basis) were associated with marine lipid supply in the diet (Franklin et al., 1999; Shingfield et al., 2003; Rego et al., 2005) or in the abomasum (Dallaire et al., 2014).

Although induction of MFD occasionally may be a management tool, as in some situations of negative energy balance and in markets where milk production is regulated by a quota system based on milk fat (Bauman et al., 2011), most often, diet-induced MFD is unintended and is more frequently perceived as being clearly negative. This decrease in milk fat synthesis not only results in a direct economic loss, but is also associated with a reduction in feed conversion efficiency (Hostens et al., 2011), sometimes provoked by the occurrence of subacute ruminal acidosis (Enemark, 2008). In extreme cases of MFD, the profile of *trans* 18:1 and 18:2 isomers in milk fat resembles that of partially hydrogenated plant oils (Shingfield et al., 2009), which have been identified as detrimental for human health (Mensink et al., 2003). Indeed, under MFD conditions, a shift occurs in milk FA profile from *trans*-11 18:1 as the major *trans* FA toward increased proportions of *trans*-10 18:1 (Conte et al., 2018). Epidemiological studies indicated that industrial *trans* FA, which are also enriched in *trans*-10 18:1, have a negative effect on serum cholesterol and lipoprotein metabolism, thereby increasing the risk for coronary heart disease (Kuhnt et al., 2016). Nevertheless, to the best of our knowledge, no human intervention study has been performed with *trans*-10 18:1-containing milk (or dairy products), and only 2 animal studies have compared milk or butter enriched in *trans*-10 18:1 or *trans*-11 18:1. Roy et al. (2007) observed increased total cholesterol and low-density lipoprotein cholesterol concentrations in plasma and increased lipid deposition in the aorta of rabbits supplemented with *trans*-10 18:1 compared with *trans*-11 18:1-enriched butter. Furthermore, plasma triacylglycerides concentrations tended to increase in rats treated with *trans*-10 18:1-enriched milk fat, whereas milk fat containing *trans*-11 18:1 and *cis*-9,*trans*-11 CLA provoked the opposite (Anadón et al., 2010). Although those animal studies showed a potential negative risk of *trans*-10 18:1-containing dairy products for human health, extrapolation of findings from animal studies to humans has to be made with caution. Some feeding strategies that increase *trans*-10 18:1 proportion in dairy products may induce larger increments in *trans*-11 18:1 and *cis*-9,*trans*-11 CLA or other bioactive FA that counteract the potentially negative effects of *trans*-10 18:1, as suggested in a hamster model (Lock et al., 2005). As such, further research is required to evaluate the effects of milk from animals under extreme MFD conditions on human health. Above this human health consequence, MFD is often associated with modified ruminal fermentation and frequently considered an indicator for impaired animal health (i.e., ruminal acidosis) and reduced ruminal efficiency (Enemark, 2008; Harvatine et al., 2009), thereby detrimentally affecting

animal welfare. Hence, MFD is an undesirable situation both from an economic perspective, as well as from an animal welfare and human health perspective.

### DIETS ASSOCIATED WITH MFD

Diets causing MFD can be divided into 2 broad groups: (1) diets rich in rapidly fermentable carbohydrates (**RFCH**), low in physically effective fiber (**peNDF**), or both, and (2) diets supplemented with UFA, especially marine lipids containing eicosapentaenoic acid (**EPA**, 20:5n-3) and docosahexaenoic acid (**DHA**, 22:6n-3; Bauman and Griinari, 2003).

#### *Diets Rich in RFCH, Low in peNDF, or Both*

Increasing the fermentability of a cow's diet has been frequently used to meet the high energy requirements of high-yielding dairy cows. Nevertheless, increasing the fermentability of the diet does not only increase the milk production, but it also affects the rumen environment, potentially inducing MFD. The most common type of diet within this group is a high-grain/low-forage diet, particularly when rapidly fermentable starch sources are employed, such as wheat grain or high-moisture corn (Jurjanz et al., 2004; Weimer et al., 2010). However, diets in which the fiber content is adequate but the peNDF content is inadequate (e.g., a pelleted fiber source, such as pelleted alfalfa) could fall into this group because of the rapid fermentation of these fiber sources, which reduces the ability to maintain normal rumen function (Colman et al., 2013). It is also possible to observe MFD in grazing animals, especially in early spring (Rivero and Anrique, 2015). Young pastures contain a significant amount of UFA, in addition to high concentrations of sugars and soluble fiber, resulting in a low peNDF content. Selection by grazing cows against the fiber content (Jacobs et al., 1999) might decrease the relative intake of peNDF even more. Furthermore, diet fermentability may interact with other ingredients of the ration, and feeding rapidly fermentable diets together with PUFA-rich supplements from plant sources, soybean, and sunflower, is known to increase the risk of MFD (Ventto et al., 2017). On the other hand, literature on this topic suggests that RFCH diets with or without plant lipids inhibit de novo FA synthesis but rarely impair total milk fat synthesis in small ruminants (Mele et al., 2006; Gómez-Cortés et al., 2008; Nudda et al., 2014), and very few reductions in milk fat concentration have been found in ewes and does under those feeding conditions (Zhang et al., 2006; Bernard et al., 2012; Shi et al., 2015). These results may be explained by the ability of small ruminants to counteract the inhibition in de novo FA synthesis

by concomitant increments in preformed FA secretion (Toral et al., 2020).

Diets rich in RFCH or low in peNDF often result in subacute ruminal acidosis, characterized by a reduced rumen pH for several hours per day (Plaizier et al., 2008). Reduced rumen pH was proposed to induce MFD by alterations in rumen fermentation (Bauman and Griinari, 2001). However, decreases in milk fat content upon increased diet fermentability are not always associated with reduced rumen pH (Colman et al., 2010; Ramirez-Ramirez et al., 2015; Dewanckele et al., 2019b). Furthermore, data from 28 studies in dairy cows (Figure 1A, B) show that rumen pH is related to neither milk fat content ( $r^2 = 0.190$ ) nor milk fat yield ( $r^2 = 0.070$ ). This suggests that a low rumen pH is not the main determinant of MFD.

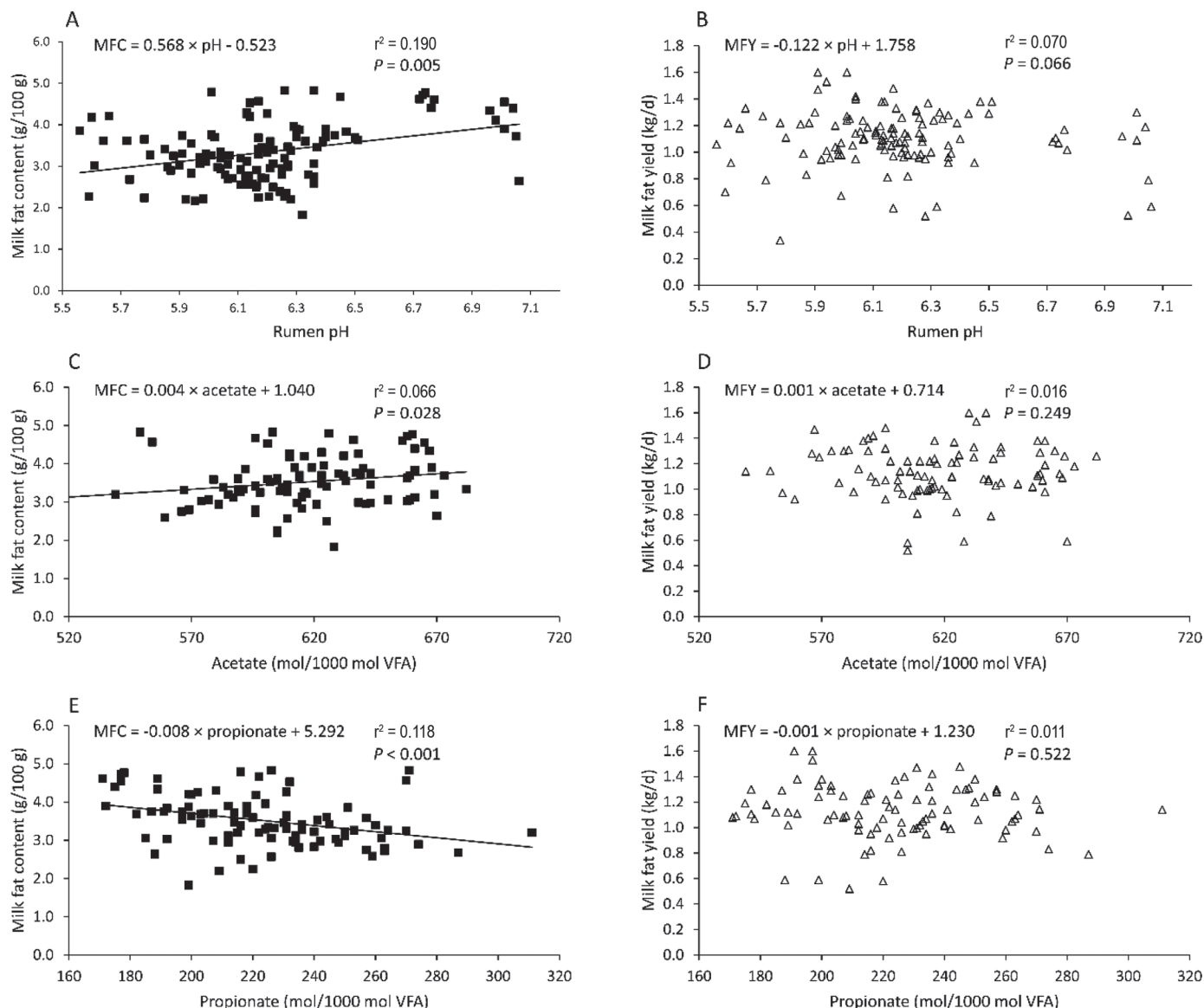
Considering the importance of acetate as a carbon source for milk fat synthesis and the observed shift in the rumen VFA profile toward less acetate and more propionate with diets rich in RFCH, low in peNDF, or both, acetate deficiency or propionate overflow might induce MFD. Bauman and Griinari (2001) reviewed the acetate deficiency theory and concluded there was little support for it. However, more recent articles have renewed the interest in the potential effect of shifts in VFA on mammary lipogenesis. For example, Urrutia et al. (2019) observed increased milk fat yield (+90 g/d) and concentration (+0.2 percentage units) upon dietary supplementation with sodium acetate at 2.9% of diet DM. In the study by Maxin et al. (2011), ruminal infusion of 800 g/d of propionate to dairy cows reduced the milk fat content and yield by 7.8 and 9.8%, respectively (mainly through changes in preformed FA), whereas ruminal infusion of the same amount of acetate increased the milk fat content by 6.5% (mainly through promotion of de novo FA synthesis). Nevertheless, data from 28 recent studies (Figure 1C–F and Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-17662>) revealed that rumen proportions of acetate or propionate do not affect milk fat content or yield ( $r^2 \leq 0.118$ ). Although these studies relied on acetate and propionate proportions rather than productions, the lack of any relation suggests that shifts in rumen VFA, induced by increasing RFCH, are not a major cause of the associated reductions in milk fat content and yield. Moreover, the decrease in milk fat content provoked by propionate infusion (Maxin et al., 2011) particularly affected preformed milk FA, in contrast to the decreased de novo synthesized milk FA during diet-induced MFD.

#### *Diets Supplemented with UFA*

Plant lipids are added to the ration of dairy ruminants to increase the energy density, modify milk fat

composition, or both (Palmquist and Jenkins, 2017) but, as mentioned above, inclusion of UFA-rich seeds and oils, particularly in combination with RFCH-rich diets, typically cause MFD in cows (Rico and Harvatine, 2013; Saliba et al., 2014; Ventto et al., 2017). Supplementation with marine lipids is not a common practice on commercial farms, although it has drawn attention in dairy science in recent years (Shingfield et al., 2003; Loor et al., 2005; Pirondini et al., 2015).

Fish oils and lipids from marine mammals and marine algae are characterized by the presence of 2 PUFA: EPA and DHA. Marine lipids have been added to ruminant diets in an attempt to increase the concentrations of human health-promoting PUFA in milk and meat (Lock and Bauman, 2004). Indeed, supplementation with products enriched in EPA and DHA to dairy cows resulted in lower rumen concentrations of SFA, whereas the concentrations of MUFA and PUFA increased



**Figure 1.** Relationship between milk fat content (MFC; A, C, E) or yield (MFY; B, D, F) and rumen pH (A, B), proportion (mol/1,000 mol of VFA) of acetate (C, D), or propionate (E, F). Square = milk fat content; triangle = milk fat yield. Data derived from 28 studies (Bhandari et al., 2008; Boeckaert et al., 2008; Enjalbert et al., 2008; Gozho and Mutsvangwa, 2008; Hristov et al., 2009, 2011a,b; Iqbal et al., 2009; Longuski et al., 2009; Oelker et al., 2009; Agle et al., 2010; Côrtes et al., 2010; Zhang et al., 2010; Dschaak et al., 2011; Martel et al., 2011; Mathew et al., 2011; Benchaar et al., 2012, 2015; Brask et al., 2013; Hassanat et al., 2013; Cruywagen et al., 2015; Pirondini et al., 2015; Rico et al., 2015; Van Gastelen et al., 2015; Ramirez Ramirez et al., 2016a,b; Szczechowiak et al., 2016; Lopes et al., 2017).



(Boeckaert et al., 2007), resulting in a higher ratio of UFA to SFA in milk fat (Boeckaert et al., 2008; Kairenius et al., 2015). Consistent results have been reported in small ruminants (Bernard et al., 2015; Toral et al., 2016a,b). It should be noted here that the decrease in SFA due to marine oil supplementation is mostly caused by a decrease in 18:0 (Boeckaert et al., 2007), which is a neutral FA in regard to human health. Nevertheless, the increase in UFA might be positive, as several UFA have been shown to beneficially affect human health. For example, n-3 PUFA, such as EPA, DHA, and 18:3n-3, play a beneficial role in the prevention of cardiovascular disease (Jung et al., 2008), and increasing consumption of n-6 (e.g., 18:2n-6) and n-3 PUFA contributes to the prevention of age-related weight gain (Liu et al., 2018). Furthermore, *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA have been shown to affect immune function and to have protective effects against cancer, obesity, diabetes, and atherosclerosis (Yang et al., 2015). Although the dietary intake of those health-promoting UFA through milk is low, preventive benefits of milk consumption against several diseases have been described (Kuhnt et al., 2016).

Supplementation of ruminant diets with marine lipids does not only increase human health-promoting FA in milk, but might also result in MFD (Boeckaert et al., 2008; Kairenius et al., 2015). In contrast to interspecies differences in the response to high RFCH/low peNDF diets, marine lipid-induced MFD has consistently been described in bovine, ovine, and caprine, indicating that the underlying mechanisms of the 2 MFD types might differ (Carreño et al., 2016; Frutos et al., 2017; Fougère et al., 2018). Indirect comparisons among studies in the literature tended to suggest that goats and sheep were less prone than cattle to detrimental effects of marine lipids on mammary lipogenesis (Papadopoulos et al., 2002; Boeckaert et al., 2008; Bernard et al., 2015). Nevertheless, the type of basal diet might play a role. The forage in the ration of small ruminants is usually hay or grass silage, whereas lactating dairy cows often receive maize silage. When cows and goats were fed exactly the same basal diet (i.e., grass hay and concentrates) in direct comparative studies, similar milk fat responses were observed after marine lipid supplementation (Toral et al., 2015; Fougère et al., 2018), supporting that the type of diet may indeed play a role.

Marine lipids inhibit the final step of biohydrogenation to 18:0 in the rumen of cows, sheep, and goats (e.g., Boeckaert et al., 2007; Frutos et al., 2018; Dewanckele et al., 2018), resulting in a shortage of 18:0 (melting point = 69.7°C) for mammary uptake that would constrain the synthesis of *cis*-9 18:1 (melting point = 16.0°C) by  $\Delta^9$ -desaturation (Gama et al., 2008). In addition, marine lipids increase the milk fat proportion of *trans*

18:1 isomers (Loor et al., 2005; Kairenius et al., 2015), which have a higher melting point ( $52 \pm 2.1^\circ\text{C}$ ) than their equivalent *cis* isomers ( $33 \pm 4.2^\circ\text{C}$ ; Gunstone et al., 1994; LipidBank database, <http://www.lipidbank.jp>, Japanese Conference on the Biochemistry of Lipids). These alterations in milk FA profile upon marine oil supplementation, which have been described across ruminant species (Boeckaert et al., 2008; Carreño et al., 2016; Fougère et al., 2018), might challenge the ability of the mammary gland to maintain milk fat melting point below body temperature (Timmen and Patton, 1988). An extension of the biohydrogenation theory postulated that increased milk fat melting point may impair the capacity to achieve an adequate fluidity for milk fat secretion, which might account for this type of MFD (Shingfield and Grinari, 2007). Nevertheless, supplementation of 18:0 to the diet of lactating sheep did not alleviate fish oil-induced MFD in the experiment of Toral et al. (2016b), challenging the theory of a shortage of 18:0. Surprisingly, the combination of fish oil and 18:0 further aggravated the *trans*-10 shift in biohydrogenation pathways compared with the addition of fish oil alone (Toral et al., 2016b). Also in cows, supplementation of a blend of fish oil in combination with either a fat rich in 18:0 or a plant oil high in linolenic acid oil resulted in similar responses in milk fat concentration and yield, as well as ruminal *trans*-10 18:1 proportions (AbuGhazaleh et al., 2003). Accordingly, and contrary to the expectations based on separate supplementations of PUFA- or 18:0-rich lipid sources (Benchaar et al., 2012; Boerman et al., 2017; Kairenius et al., 2018), their combination with fish oil resulted in similar disturbances in biohydrogenation.

Although shifts in rumen VFA do not seem to be a major cause of reduced milk fat content and yield in Figure 1C-F and Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-17662>), Frutos et al. (2018) observed lower acetate concentrations in rumen fluid of sheep displaying strong MFD compared with sheep showing mild MFD, upon dietary supplementation with 2% fish oil. Trials conducted in bovine and caprine animals (in vitro and in vivo) have also shown marine lipid-induced reductions in ruminal acetate concentrations and decreases in acetate:propionate ratio (AbuGhazaleh and Ishlak, 2014; Vlaeminck et al., 2015; Zhu et al., 2016). Perhaps, shifts in VFA and putative consequences on de novo synthesis and preformed FA uptake play a more important role in marine lipid-induced MFD compared with RFCH-induced MFD, which warrants additional and targeted research. Results from recent studies would also recommend re-evaluating the relevance of the inhibition in preformed FA uptake in marine lipid-induced MFD (Dallaire et al., 2014; Frutos et al., 2017; Toral et al., 2020).

## BIOHYDROGENATION THEORY

The biohydrogenation theory established that diet-induced MFD involves an interrelationship between rumen digestive processes and mammary tissue metabolism (Bauman and Griinari, 2003; Harvatine et al., 2009). Diets known to induce MFD alter rumen biohydrogenation pathways of dietary PUFA toward the formation of specific FA intermediates. After absorption in the duodenum and transfer to the mammary gland via the blood stream, some of those biohydrogenation intermediates might inhibit milk fat synthesis. First, the ruminal metabolism of dietary lipids will briefly be explained. Second, a short overview will be given of the main rumen biohydrogenation pathways, after which the association between specific biohydrogenation intermediates and MFD will be discussed in more detail.

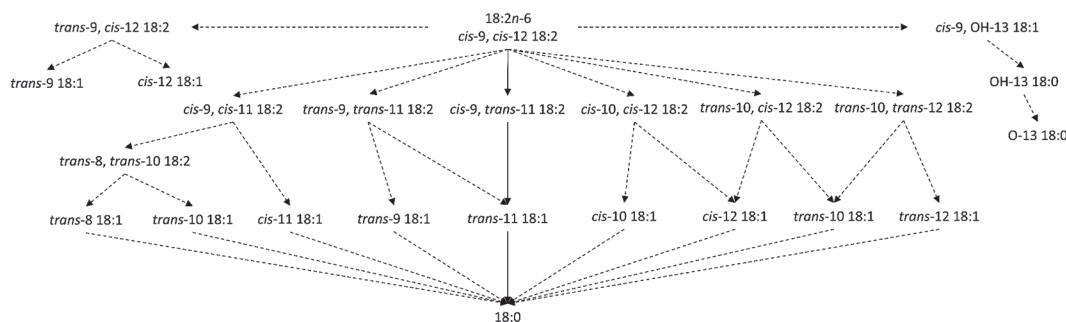
### Lipid Metabolism in the Rumen

The diet of lactating dairy cows typically contains 4 to 5% crude fat (on a DM basis), corresponding to about 2.3 to 2.5% total FA (Palmquist and Jenkins, 2003; Schmidely et al., 2008). Primary sources of lipid in the ruminant diet are forages and concentrates, which mainly contain 18-carbon UFA (i.e.,  $\alpha$ -linolenic acid, 18:3n-3; linoleic acid, 18:2n-6; and oleic acid, *cis*-9 18:1; Ferlay et al., 2017). However, the lipid content can be increased by the use of fat supplements. The major lipid class of forages is glycolipids, whereas the majority of lipids in concentrates is present in the form of triacylglycerides. Following ingestion, dietary lipids are hydrolyzed, and the nonesterified FA are released into the rumen. Then, 18:3n-3, 18:2n-6, and *cis*-9 18:1 are converted to SFA via a *cis-trans* isomerization to *trans* FA intermediates, followed by hydrogenation of the double bonds (Harfoot and Hazlewood, 1997). This process is called biohydrogenation.

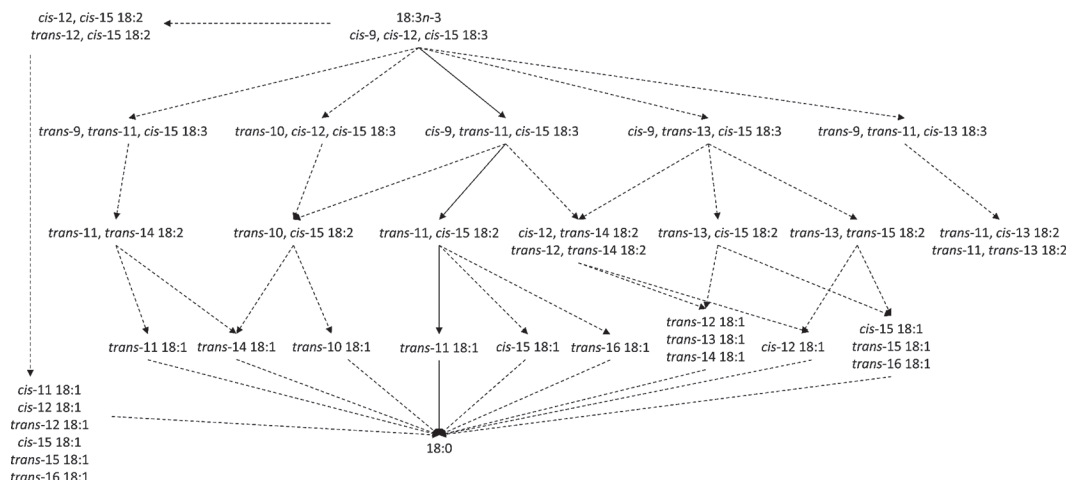
## Main Rumen Biohydrogenation Pathways

Numerous in vivo and in vitro studies have enabled several ruminal biohydrogenation pathways of 18:2n-6, 18:3n-3, and *cis*-9 18:1 to be elucidated. Under normal rumen conditions, 18:2n-6 is mainly isomerized to *cis*-9, *trans*-11 CLA, which is further hydrogenated to *trans*-11 18:1 and ultimately to 18:0 (Figure 2). The major biohydrogenation pathway of 18:3n-3 involves *cis*-9, *trans*-11, *cis*-15 conjugated linolenic acid (CLnA), *trans*-11, *cis*-15 18:2 and *trans*-11 18:1 as intermediates (Figure 3), whereas the major part of *cis*-9 18:1 is directly hydrogenated to 18:0 in the rumen (Figure 4). However, ruminal biohydrogenation of 18:2n-6, 18:3n-3, and *cis*-9 18:1 might also result in the formation of several other minor FA intermediates, such as *trans*-9, *trans*-11 CLA, *trans*-10 18:1, and *cis*-12 18:1 (Figure 2–4). In addition to the pathways shown in Figures 2–4, alternative pathways might exist, as supported by the identification of additional biohydrogenation intermediates in recent in vitro experiments using stable isotopes in cows (deuterium oxide; Honkanen et al., 2016) and sheep ( $^{13}\text{C}$ -labeled FA; Toral et al., 2018b, 2019).

Biohydrogenation is extensive, resulting in 18:0 being the major FA leaving the rumen (Shingfield and Wallace, 2014). However, the reduction of unsaturated 18-carbon FA to 18:0 in the rumen is incomplete, and numerous 18:1, 18:2, and 18:3 intermediates accumulate. Diets known to induce MFD would often result in greater amounts of particular 18:1 intermediates and minor amounts of 18:2 and 18:3 intermediates escaping the rumen compared with normal situations. Plant lipids rich in C18 UFA provide the substrates for the direct increase in 18:1 production in the rumen (Jenkins et al., 2008; Shingfield et al., 2011). As mentioned above, dietary supplementation of marine lipids rich in EPA and DHA consistently inhibited the final biohydrogenation step to 18:0 in cows, sheep, and goats



**Figure 2.** Pathways of ruminal 18:2n-6 metabolism (based on Shingfield and Wallace, 2014). Arrows with solid lines highlight the major biohydrogenation pathway, and arrows with dashed lines describe the formation of minor intermediates, under physiologically normal conditions in the rumen.



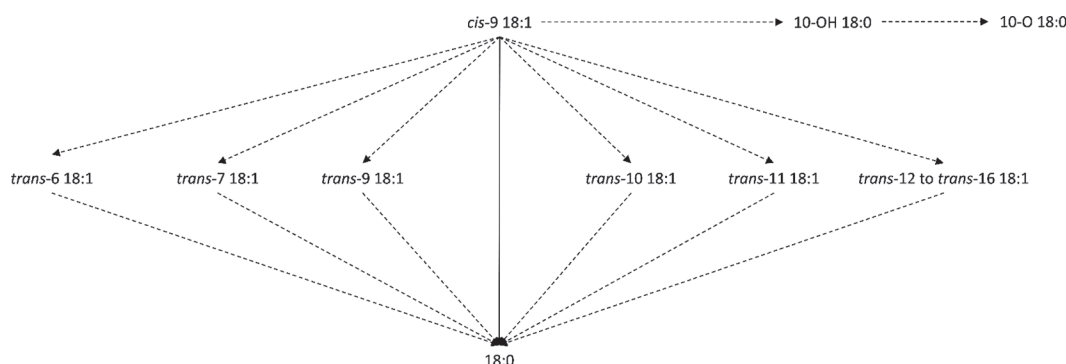
**Figure 3.** Pathways of ruminal 18:3n-3 metabolism (based on Ferlay et al., 2017). Arrows with solid lines highlight the major biohydrogenation pathway, and arrows with dashed lines describe the formation of minor intermediates, under physiologically normal conditions in the rumen.

(Shingfield et al., 2012; Zhao et al., 2016; Dewanckele et al., 2018), resulting in the indirect accumulation of 18:1 isomers, although other steps may also be affected. Additionally, in this case, some biohydrogenation intermediates of very long-chain PUFA with 20 or 22 carbons are accumulating. Moreover, decreases in rumen pH below 6.0 lowered the extent of 18:2n-6 and 18:3n-3 isomerization and inhibited the final reduction to 18:0 in vitro in cattle (Troegeler-Meynadier et al., 2006; Fuentes et al., 2009).

## Biohydrogenation Intermediates Associated with MFD

**Trans-10,cis-12 CLA.** In several studies, diets known to induce MFD were associated with increased proportions of *trans*-10,*cis*-12 CLA in the rumen (Toral et al., 2016a) or in milk (Enjalbert et al., 2008; Rico and Harvatine, 2013) of dairy cows. This was confirmed

by Conte et al. (2018) using a canonical discriminant analysis. Although such association does not necessarily support a causal effect, evidence of an inhibitory role of *trans*-10,*cis*-12 CLA on milk fat synthesis has been provided by its postruminal infusion (Baumgard et al., 2000), which decreased milk fat synthesis in the lactating cow in a dose-dependent manner (Baumgard et al., 2001). An antilipogenic effect of this CLA isomer was subsequently demonstrated in goats and sheep as well (Lock et al., 2008; Hussein et al., 2013). This coincided with a decrease in the mRNA abundance of lipogenic genes coding for key enzymes involved in milk fat synthesis in the 3 species (Baumgard et al., 2002; Peterson et al., 2003; Hussein et al., 2013; Zhang et al., 2018). However, as reported before by Shingfield and Griinari (2007), diet-induced MFD in cows occurs when the milk fat proportion of *trans*-10,*cis*-12 CLA is very low ( $\leq 0.16$  g/100 g of FA; Table 1, Figure 5A and E). In marine lipid-induced MFD, most often no



**Figure 4.** Pathways of ruminal *cis*-9 18:1 metabolism (based on Shingfield and Wallace, 2014). Arrows with solid lines highlight the major biohydrogenation pathway, and arrows with dashed lines describe the formation of minor intermediates, under physiologically normal conditions in the rumen.

change in *trans*-10,*cis*-12 CLA concentration is observed (Vahmani et al., 2013; Kairenius et al., 2015; Toral et al., 2015; Fougère et al., 2018). Furthermore, regression analysis, based on 23 studies (Figure 5A, C, and E), reveals that the milk fat proportion of *trans*-10,*cis*-12 CLA explained only 27 and 29% of the variation in milk fat content and yield, respectively. Similarly, a recent meta-analysis downplayed the role of this CLA isomer in explaining marine lipid-induced MFD in ovine (Toral et al., 2020). Overall, this suggests that other biohydrogenation intermediates might also exert antilipogenic effects, or that other factors and mechanisms are involved in the regulation of milk fat synthesis. However, it should be noted here that the very low *trans*-10,*cis*-12 CLA concentrations observed in milk fat complicate regression analysis, and the results observed in Figure 5 (A, C, and E) should be interpreted with caution.

**Trans-10 18:1.** Shingfield and Griinari (2007) indicated that diets causing lower milk fat concentration and yield are consistently associated with an increase in the milk fat *trans*-10 18:1 proportion, which has been confirmed by regression analysis of studies with dairy cows (Figure 5B, D, and F) and canonical discriminant analysis (Conte et al., 2018). However, in their recent meta-analysis, Toral et al. (2020) split data of dairy ewes into a subset with and without MFD. Similar shifts in *trans*-10 18:1 were observed in both subsets, excluding a major role of this FA in diet-induced MFD in this species. Hence, the potential role of this isomer in diet-induced MFD is equivocal.

Direct evidence of the potential role of this isomer can be provided by postprandial infusion studies or in vitro assessments with cell lines. Downregulated expression of *FASN*, *SCD*, and *SREBF1* upon incubation of mammary epithelial cells with *trans*-10 18:1 (Kadegowda et al., 2009) supports its potential antilipogenic properties. Nevertheless, a direct effect of *trans*-10 18:1 on milk fat synthesis was denied in a first report (Lock et al., 2007). In that study, abomasal infusion of 42.6 g of pure *trans*-10 18:1/d for 4 d increased the milk fat *trans*-10 18:1 concentration from 0.47 to 1.11 g/100 of g of FA, but no decrease in milk fat secretion was observed. This lack of effect on milk fat content or yield was interpreted as strong evidence that *trans*-10 18:1 does not inhibit milk fat synthesis during MFD. However, the average transfer efficiency of the abomasally infused *trans*-10 18:1 into milk fat was only 15% (Lock et al., 2007), whereas Shingfield and Griinari (2007) reported a mean transfer efficiency from the abomasum into milk fat of 32.1% for this intermediate. Furthermore, the mean milk fat proportion of *trans*-10 18:1 is higher (1.91 g/100 of g of FA; Table 1) than the proportion obtained by Lock et al. (2007) upon ab-

omasal infusion (1.11 g/100 g of FA). As such, the difference in milk fat proportion of *trans*-10 18:1 between the control and the *trans*-10 18:1 infusion might have been too small to induce significant changes between both treatments (expected decrease of 0.16 g of milk fat per 100 g of milk, based on the equation presented in Figure 5B). Indeed, a higher milk fat *trans*-10 18:1 proportion (4.37 g/100 g of FA) upon abomasal infusion decreased the milk fat content and yield by 21.3 and 19.5%, respectively, in the study by Shingfield et al. (2009). Nevertheless, in that study, a mixture of 18:1 FA methyl esters was used, containing (g/100 g of FA) *cis*-9 18:1 (9.45), *cis*-12 18:1 (3.35), *trans*-10 18:1 (37.3), *trans*-11 18:1 (37.4), and *trans*-12 18:1 (2.66) as major isomers. As such, MFD could not be directly attributed to *trans*-10 18:1. To our knowledge, no other abomasal infusion studies using relatively pure *trans*-10 18:1 were performed. This gap in the literature also concerns small ruminants, for which high milk *trans*-10 18:1 concentrations have been reported in the absence of clear MFD (Gómez-Cortés et al., 2008; Bernard et al., 2015), suggesting potential interspecies differences in the response to this FA. Interestingly, in their recent meta-analysis using data of dairy ewes, Toral et al. (2020) highlighted *trans*-10 18:1 to be consistently associated with reduced concentrations and yields of de novo synthesized milk FA. However, only in part of the studies this resulted in an overall MFD. Indeed, in cases without MFD, the decreased secretion of de novo synthesized FA seemed counteracted by increments of preformed FA.

**Cis-10,*trans*-12 CLA.** Sæbø et al. (2005) suggested that *cis*-10,*trans*-12 CLA has antilipogenic effects based on abomasal infusions of geometric isomers of 10, 12 CLA. However, in that study, *cis*-10,*trans*-12 CLA was infused in combination with *trans*-10,*cis*-12 CLA, which is known to have an antilipogenic effect (Baumgard et al., 2000; Peterson et al., 2003). Nevertheless, abomasal infusion of 4.137 g of *trans*-10,*cis*-12 CLA/d resulted in a similar milk fat decrease as compared with a combination of 1.802 g of *trans*-10,*cis*-12 CLA and 1.194 g of *cis*-10,*trans*-12 CLA/d, indicating a potential role of the latter FA isomer in MFD. No other studies showing a possible direct effect of *cis*-10,*trans*-12 CLA on milk fat synthesis have been performed since 2005. Furthermore, recent studies did not report the milk fat proportion of this CLA isomer (Table 1), potentially indicating that *cis*-10,*trans*-12 CLA is hardly observed in milk fat of dairy cows and is not a major cause of diet-induced MFD.

**Trans-9,*cis*-11 CLA.** The potential role of *trans*-9,*cis*-11 CLA in MFD was shown by Perfield et al. (2007), who abomasally infused 5 g of *trans*-9,*cis*-11 CLA/d and reduced both the milk fat content and



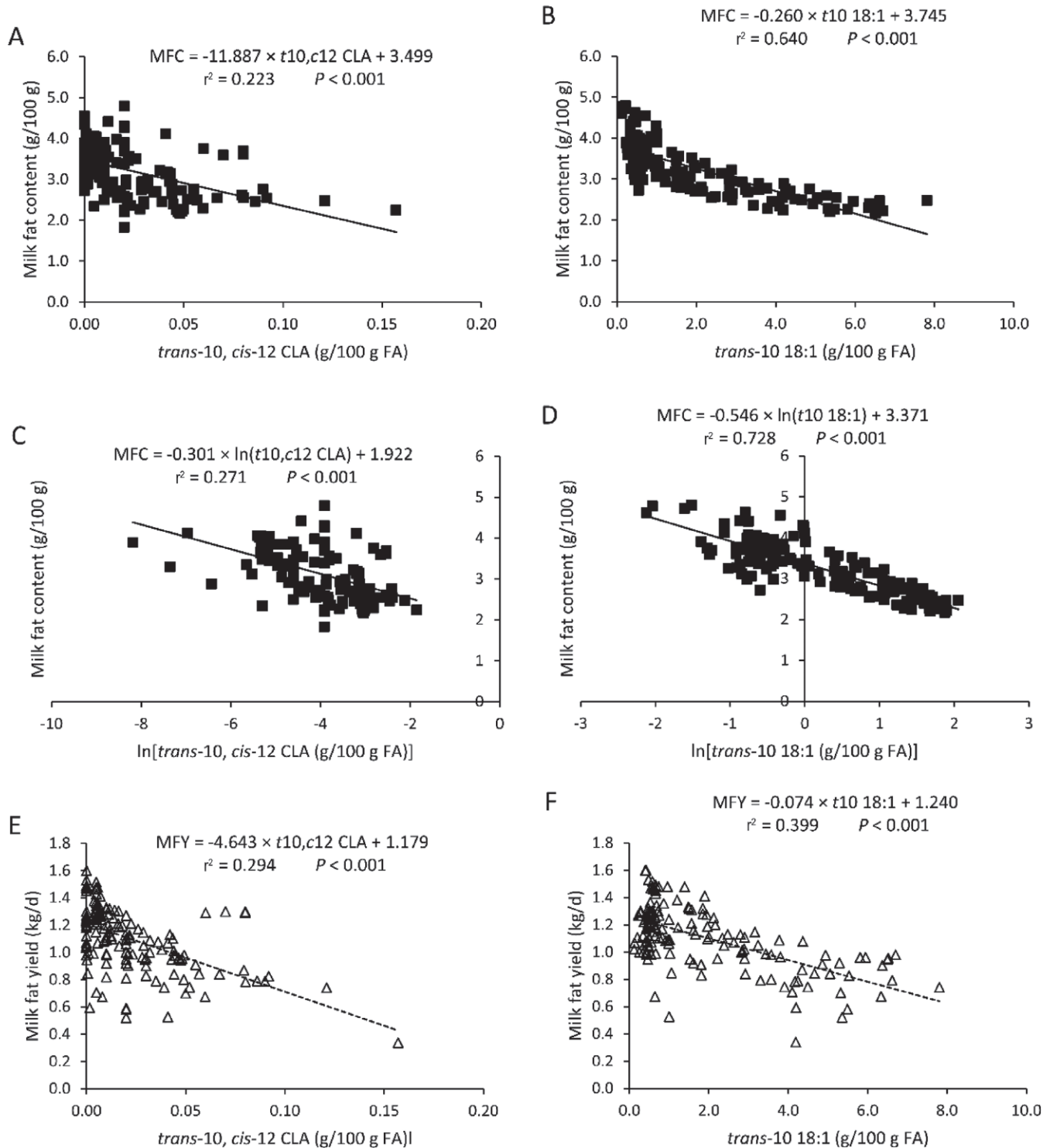
**Table 1.** Minimum, maximum, and mean milk fat yield or content, or milk fat proportion of biohydrogenation intermediates associated with milk fat depression,<sup>1</sup> as well as results of abomasal infusion studies of those intermediates

Parameter <sup>2</sup>	Reports <sup>3</sup>	Abomasal infusion						
		Minimum	Maximum	Mean	Milk fat proportion	Δ Milk fat content (%)	Δ Milk fat yield (kg/d)	Study
Milk fat								
Yield (kg/d)	34	0.34	1.60	1.11				
Content (g/100 g of milk)	34	1.83	4.83	3.33				
Fatty acid (g/100 g of fatty acid)								
c11 18:1	15	0.42	1.48	0.75				
t4 18:1	9	0.02	0.10	0.04				
t5 18:1	11	0.01	0.35	0.04				
t6,7,8 18:1	14	0.15	1.31	0.54				
t9 18:1	18	0.11	0.98	0.44				
t10 18:1	23	0.12	7.81	1.91	1.11	+0.07	-0.02	Lock et al., 2007
					4.37	-0.86	-0.236	Shingfield et al., 2009
t8, t10 CLA	4	<0.01	0.02	<0.01	0.38	-0.53	-0.112	Perfield et al., 2007
t9, c11 CLA	5	0.01	0.10	0.04	0.13	-1.06	-0.280	Baumgard et al., 2000, 2001, 2002; Sæbø et al., 2005;
t10, c12 CLA	23	<0.01	0.16	0.02	0.15	-0.72	-0.193	Perfield et al., 2006; Lock et al., 2007; Perfield et al., 2007; Shingfield et al., 2009
					0.18	-0.72	-0.141	
					0.18	-1.00	-0.296	
					0.21	-1.06	-0.199	
					0.23	-1.67	-0.503	
					0.32	-1.10	-0.257	
					0.39	-1.12	-0.372	
					0.49	-1.33	-0.298	
					0.70	-1.39	-0.389	
t10, t12 CLA	4	<0.01	0.03	<0.01	0.11	-0.09	-0.028	Perfield et al., 2006

<sup>1</sup>Data derived from 34 studies (Blandari et al., 2008; Boeckaert et al., 2008; Enjalbert et al., 2010; Zhang et al., 2010; Cortes et al., 2010; Dschaak et al., 2011; Hristov et al., 2011a,b; Martel et al., 2011; Mathew et al., 2011; Benchaar et al., 2012, 2015; He et al., 2012; Brask et al., 2013; Hassanat et al., 2013; Rico and Harvatine, 2013; Boerman et al., 2014; Cruywagen et al., 2015; Kairenius et al., 2015; Pirondini et al., 2015; Rico et al., 2015; Toral et al., 2015; Van Gastelen et al., 2015; Ramirez Ramirez et al., 2016a,b; Szczechowiak et al., 2016; Lopes et al., 2017; Rico et al., 2017).

<sup>2</sup>t = *trans*; c = *cis*.

<sup>3</sup>Number of reports in which the milk fat proportion of the respective intermediate was analyzed.



**Figure 5.** Relationship between milk fat content (MFC; A–D) or yield (MFY; E–F) and milk fat proportion (g/100 g; FA = fatty acids) of *trans*-10,*cis*-12 CLA (A, C, E) or *trans*-10 18:1 (B, D, F), either (C, D), or not (A, B, E, F), after a natural log-transformation of the milk fat proportion data of *trans*-10,*cis*-12 CLA or *trans*-10 18:1. Square = milk fat content; triangle = milk fat yield; solid line = linear relation with milk fat content; dashed line = linear relation with milk fat yield. The linear relation between the natural log-transformed proportions of both FA and milk fat yield did not result in an improvement of the relation as compared with the non-transformed data (data not shown). Data derived from 23 studies (Boeckaert et al., 2008; Enjalbert et al., 2008; Hristov et al., 2009, 2011a,b; Oelker et al., 2009; Dschaak et al., 2011; Martel et al., 2011; Mathew et al., 2011; Benchaar et al., 2012; He et al., 2012; Rico and Harvatine, 2013; Boerman et al., 2014; Kairenius et al., 2015; Pirondini et al., 2015; Rico et al., 2015; Toral et al., 2015; Van Gastelen et al., 2015; Ramirez Ramirez et al., 2016a,b; Szczechowiak et al., 2016; Lopes et al., 2017; Rico et al., 2017).

yield by 15%. Nevertheless, in that study, the milk fat proportion of *trans*-9,*cis*-11 CLA was 0.38 g/100 g of FA, which is much higher than the maximum proportion of 0.10 g/100 g of FA presented in Table 1. It is not clear whether milk fat proportions of *trans*-9,*cis*-11 CLA below or equal to 0.10 g/100 g of FA would also reduce milk fat synthesis.

**Other FA.** An in vitro study using  $^{13}\text{C}$ -labeled 18:3n-3 and rumen inoculum from sheep speculated on the potential contribution of 18:3 isomers (of which some were unidentified) in marine lipid-induced MFD (Toral et al., 2019). Gervais and Chouinard (2008) investigated the effect of intravenous infusion of *cis*-9,*trans*-11,*cis*-15 18:3 and *cis*-9,*trans*-13,*cis*-15 18:3 on milk fat synthesis in lactating dairy cows. However, their study offered no support for a role of those isomers in MFD, although this does not exclude the potential involvement of other 18:3 isomers.

Studies conducted in cows, ewes, and does have shown that MFD accompanied increases in milk fat or omasal digesta proportions of other 18-carbon FA, such as *cis*-11 18:1, *trans*-4 to *trans*-9 18:1, *trans*-8,*trans*-10 CLA, *trans*-10,*trans*-12 CLA, *trans*-10,*cis*-15 18:2, and 10-oxo-18:0 (Carreño et al., 2016; Kairenius et al., 2015; Ventto et al., 2017; Leskinen et al., 2019). Their origin is likely different and would not always be associated with biohydrogenation. For example, the increased milk fat proportion of *cis*-11 18:1 under fish oil-induced MFD conditions (Shingfield et al., 2003; Kairenius et al., 2015) might result from its supply of marine lipids and the concomitant inhibition of 18:1 saturation, rather than from an increased production in the rumen through C18 biohydrogenation. This hypothesis is supported by the in vitro incubation of  $^{13}\text{C}$ -labeled UFA with rumen inoculum from cows (Klein and Jenkins, 2011) or from sheep adapted or not to fish oil consumption (Toral et al., 2018b, 2019). As such, PUFA originating directly from fish oil might contribute, at least in part, to MFD. Indeed, abomasal infusion of 406 g of fish oil/d resulted in a modest decrease in milk fat content and yield (12 and 17%, respectively) in the experiment of Dallaire et al. (2014), which was accompanied with an increased milk fat proportion of *cis*-9 16:1, *cis*-11 18:1, EPA, and DHA, in accordance with Looor et al. (2005). Burns et al. (2012) showed that *cis*-11 18:1 lowered lipogenesis and *FASN* expression during incubation with bovine adipocytes. However, postruminal infusion of a mixture of 18:1 isomers containing *cis*-11 18:1 (12.50 g/100 g of FA) had no effect on milk fat synthesis in cows (Shingfield et al., 2007), which could have been related to an imperfect postruminal infusion resulting in a milk fat proportion of *cis*-11 18:1 that might have been too low to induce MFD. Because examining the effect of

*cis*-11 18:1 was not the objective of that study, the proportion of this isomer in milk fat was not measured, and no conclusion can be drawn about the potential involvement of *cis*-11 18:1 in MFD. In addition to *cis*-11 18:1, *cis*-9 16:1 has been shown to inhibit in vitro adipogenesis in bovine adipocytes (Burns et al., 2012) and to reduce intramuscular adipocyte size and lipid content in sheep (Duckett et al., 2014), whereas EPA decreased the mRNA abundance of lipogenic genes, such as *SREBF1*, *SCAP*, *INSIG1*, and *LPL*, in bovine mammary epithelial cells (Kadegowda et al., 2009).

In contrast to the study mentioned above, in which MFD accompanied increased omasal digesta proportions of *trans*-8,*trans*-10 CLA (Ventto et al., 2017), in the study of Conte et al. (2018), MFD was not associated with increased milk *trans*-8,*trans*-10 CLA concentrations. Furthermore, the milk fat proportion of this isomer is generally very low ( $\leq 0.02$  g/100 g of FA; Table 1), questioning its potential contribution in the reduction of milk fat synthesis.

Abomasal infusion of 4.615 g of *trans*-10,*trans*-12 CLA/d during 4 d did not affect the milk fat content and yield in the experiment of Sæbø et al. (2005). Unfortunately, the achieved proportion in milk fat could not be measured in that study due to overlap with other CLA isomers in the chromatogram. Perfield et al. (2006) abomasally infused 5.0 g *trans*-10,*trans*-12 CLA/d for 4 d, which resulted in a milk fat proportion of 0.11 g/100 g of FA. As in the study of Sæbø et al. (2005), no decrease in milk fat content or yield was observed. As the milk fat proportion of *trans*-10,*trans*-12 CLA is generally below 0.03 g/100 g of FA (Table 1), this isomer is probably not involved in milk fat synthesis. Accordingly, canonical discriminant analysis showed that this FA was not associated with MFD in the experiment of Conte et al. (2018).

Based on treatment of adipocytes with 70  $\mu\text{M}$  *trans*-10,*cis*-15 18:2, Vahmani et al. (2016) suggested that this isomer does not exert anti-adipogenic effects. However, it is unclear whether this FA isomer has antilipogenic effects in the mammary gland. Furthermore, very few experiments included in this review reported *trans*-10,*cis*-15 18:2 concentrations, which might be the result of co-elution with *trans*-11,*cis*-15 18:2 during analysis with standard columns (CP-Sil 88 and SP-2560) in GC (Alves and Bessa, 2014). The few studies that reported this isomer indicated a rather low concentration in omasal digesta and milk fat (0.15 and 0.017 g/100 g of FA, respectively, under RFCH-induced MFD conditions; Ventto et al., 2017; Leskinen et al., 2019).

Several recent in vivo studies have observed a positive association between MFD and the milk fat proportion of oxo-FA, particularly 10-oxo-18:0, in cows, ewes

and does fed fish oil (Bernard et al., 2015; Kairenius et al., 2015; Carreño et al., 2016). Those increments in 10-oxo-18:0 may derive from alterations in the relative contribution of specific pathways of ruminal *cis*-9 18:1 metabolism, as suggested in an in vitro study with rumen inoculum of sheep adapted to fish oil consumption (Toral et al., 2018b). Overall, these observations support the potential involvement of oxo-FA, and of 10-oxo-18:0 in particular, in marine lipid-induced MFD. Nevertheless, further research including abomasal infusion studies and lipogenic gene expression studies are required to confirm this hypothesis.

Similarly, no abomasal infusion or lipogenic gene expression studies testing the effects of *trans*-4 to *trans*-8 18:1 have been performed yet, at least not with relatively pure forms of the isomers. In contrast, some abomasal infusion studies were performed with *trans*-9 18:1 (Rindsig and Schultz, 1974; Tyburczy et al., 2008), but no effect on milk fat yield or percentage was observed, even though the milk fat proportion of *trans*-9 18:1 was higher (3.21 g/100 g of FA) in the experiment of Tyburczy et al. (2008) than the maximum proportion that is usually observed (0.98 g/100 g of FA, Table 1).

In addition to *trans* 18-carbon biohydrogenation intermediates, MFD induced by the supplementation of marine lipids is associated with the formation, ruminal accumulation and outflow of biohydrogenation intermediates with 20 or 22 carbon atoms. Although the biohydrogenation pathways of 20:5n-3 or 22:6n-3 are far from being elucidated, numerous intermediates have been identified recently both under in vivo and in vitro conditions, in cows and small ruminants and with mixed, as well as pure, rumen bacteria (Escobar et al., 2016; Jeyanathan et al., 2016; Aldai et al., 2018; Kairenius et al., 2018; Toral et al., 2018a). From the biohydrogenation pathways of 18:2n-6 and 18:3n-3, isomerization and migration of a *cis* double bond resulting in the production of monoconjugated 20:5 and 22:6 would have been expected. With the exception of the study by Aldai et al. (2018), such monoconjugated isomers compatible with the first product of 20:5n-3 or 22:6n-3 metabolism have not been identified in any of the previously mentioned studies, nor earlier studies with dairy cows, ewes, and goats fed marine lipids (Kairenius et al., 2011; Toral et al., 2012, 2016a). Accordingly, the potential role of the monoconjugated 20:5 and 22:6 is unlikely. Particularly 20- and 22-carbon FA metabolites commonly found in bovine and ovine ruminal contents might be of interest, as marine lipids are associated with dietary induced MFD across species (Toral et al., 2018a). Given the major emphasis on *trans*-10 containing 18-carbon FA in relation to dietary-induced MFD, increases in milk of some *trans*-10 containing biohydrogenation intermedi-

ates with 20- and 22 carbons (Kairenius et al., 2015) are of interest (e.g., *trans*-10,*trans*-14,*trans*-17 20:3 and *trans*-10,*trans*-16 20:2). The production of these intermediates has been confirmed during in vitro incubations with mixed rumen fluid from both cows and ewes (Toral et al., 2018a), but were not reported in other studies (Aldai et al., 2018; Kairenius et al., 2018). Nevertheless, in vitro cell culture or in vivo infusion studies with pure 20- and 22-carbon biohydrogenation isomers are lacking and hence, their biological activity in ruminants remains to be elucidated.

As opposed to previously mentioned FA, a repression of the *trans*-13/14 pathway was observed in vitro upon incubation of fish oil-adapted inoculum of sheep with <sup>13</sup>C-labeled 18:3n-3 (Toral et al., 2019). Similar results have previously been found in vivo in ewes fed fish oil (e.g., Frutos et al., 2018) and in cattle supplemented with fish oil alone or in combination with linseed oil (Shingfield et al., 2003, 2011; Kairenius et al., 2018). Although the biological implications of these shifts remain to be explored, a recent study has indicated that *trans*-11,*trans*-13 CLA induces lipogenesis in the liver of mice (Pachikian et al., 2018). In this regard, much effort has been made in exploring antilipogenic FA responsible for MFD (Bauman and Griinari, 2001), but the role of prolipogenic metabolites is less known.

### Conclusion Biohydrogenation Theory

Conte et al. (2018), who investigated the correlation between MFD and milk fat proportions of several biohydrogenation intermediates, found the highest correlation of MFD with *trans*-10 18:1 and *trans*-10,*cis*-12 CLA, whereas Ventto et al. (2017) and Leskinen et al. (2019) additionally suggested *trans*-10,*cis*-15 18:2 is involved in MFD. As discussed above, *trans*-10,*cis*-12 CLA indeed exerts antilipogenic properties, although this CLA isomer alone cannot explain all cases of MFD given its extremely low concentrations in most studies, particularly in response to marine lipids. Therefore, *trans*-10 18:1 and *trans*-10,*cis*-15 18:2 might be (partially) involved in MFD. Several experiments support the potential role of *trans*-10 18:1, although a direct effect has not been proven equivocally. Until now, no study has been performed to investigate the direct role of *trans*-10,*cis*-15 18:2 or (*trans*-10-containing) biohydrogenation intermediates with 20- and 22 carbons in milk fat synthesis. As such, abomasal infusion experiments using pure forms of these isomers are needed to confirm this hypothesis. Because those intermediates are not commercially available, this requires chemical synthesis and purification, which is an expensive process.

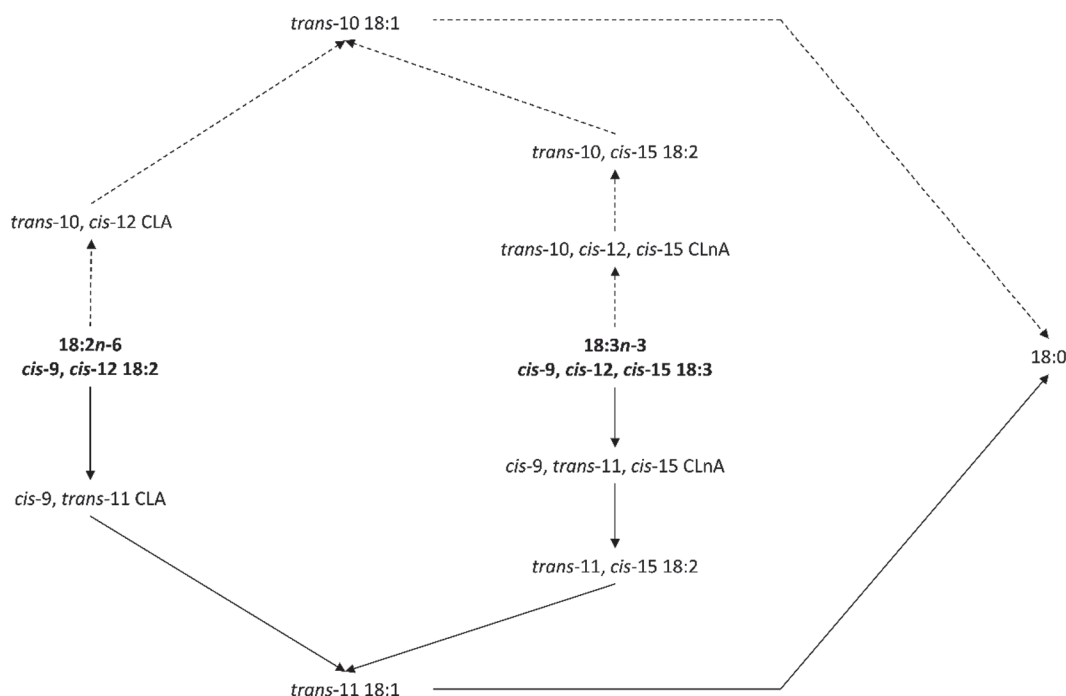


Although identified *trans*-10 intermediates only explained 34% of the milk fat content decline in the quantitative literature review of Rulquin et al. (2007), all the intermediates that show the clearest association with MFD contain a double bond in the *trans* configuration at the 10th carbon atom (from the carboxyl end). As such, it seems that circumstances that provoke a shift in the rumen biohydrogenation pathway from the formation of mainly *trans*-11 intermediates toward greater accumulation of *trans*-10 intermediates, referred to as the *trans*-11 to *trans*-10 shift (Figure 6), may induce MFD, as suggested by Conte et al. (2018). Nevertheless, the *trans*-11 to *trans*-10 shift is probably not the only mechanism explaining MFD, and other FA or metabolites or other mechanisms might also be involved in diet-induced MFD, suggesting that MFD is a multi-etiological syndrome with several causal factors. Some of these causal factors might be similar in RFCH-induced and marine lipid-induced MFD, whereas other causal factors might be more relevant to one of the 2 types of diet-induced MFD. This might also explain the interspecies differences in response to different MFD-inducing diets. Additionally, responses seem to depend on the origin of the milk FA, which warrants in-depth segregated investigations on de novo, preformed, and C16 FA secretions to further elucidate potentially com-

pensatory mechanisms in response to repressed de novo FA synthesis (Toral et al., 2020).

### RUMEN BIOHYDROGENATING BACTERIA OF 18-CARBON FA

The main members of the rumen microbial community (per mL of live liquor) are anaerobic bacteria ( $10^{10}$ ), ciliate protozoa ( $10^7$ ), and anaerobic fungi ( $10^6$ ; Jenkins et al., 2008; Buccioni et al., 2012). Bacteria, living in symbiosis with protozoa or not, are known to be mainly responsible for rumen biohydrogenation, although the contribution of protozoa and fungi is negligible (Lourenço et al., 2010; Buccioni et al., 2012). The main reason for biohydrogenation of PUFA by bacteria is thought to be reduction of the toxicity of those PUFA (Maia et al., 2007; Fukuda et al., 2009; Maia et al., 2010). The mode of action of PUFA antimicrobial activities is not yet clear, but the prime target seems to be the bacterial cell membrane and the various essential processes that occur within and at the membrane. Bacteria prefer SFA for their membrane synthesis because the double bonds present in UFA alter the shape of the molecule and disrupt the lipid bilayer structure (Keweloh and Heipieper, 1996).



**Figure 6.** Main intermediates of the primary (arrows with solid lines) and a secondary (arrows with dashed lines) biohydrogenation pathway of 18:2n-6 and 18:3n-3 in the rumen (based on Shingfield and Wallace, 2014; Ferlay et al., 2017). The secondary pathway is the result of a *trans*-11 to *trans*-10 shift. CLnA = conjugated linolenic acid.

For many years, the bacteria involved in the different steps of biohydrogenation were classified as group A and B (Kemp and Lander, 1984). Group A bacteria hydrogenate 18:2n-6 and 18:3n-3 to *trans*-11 18:1 and related isomers, whereas group B bacteria further convert those 18:1 isomers to 18:0. Nevertheless, some bacteria cannot be classified solely in 1 of those 2 groups, such as *Butyrivibrio proteoclasticus*, which not only reduces 18:1 isomers to 18:0, but also hydrogenates 18:2n-6 to 18:0 (Wallace et al., 2006). With respect to biohydrogenation intermediates associated with MFD (see above), we propose that it is more appropriate to classify the biohydrogenating bacteria into (1) bacteria involved in the *trans*-11 pathway, (2) bacteria involved in the *trans*-10 pathway of biohydrogenation, and (3) other biohydrogenating bacteria. An overview of the text described below is given in Table 2.

### Bacteria Involved in the *Trans*-11 Pathway

*Butyrivibrio fibrisolvens* was the first ruminal bacterial species found to carry out biohydrogenation of 18:2n-6 and 18:3n-3 in vitro (Kepler et al., 1966; Kepler and Tove, 1967). According to Kepler et al. (1966) and Kepler and Tove (1967), 18:2n-6 is first isomerized to *cis*-9,*trans*-11 CLA by this species, which is then further reduced to a mixture of *trans*-9 and predominantly *trans*-11 18:1, being the end products of its 18:2n-6 metabolism. However, more recent in vitro studies revealed that 18:2n-6 is not only isomerized to *cis*-9,*trans*-11 CLA by *B. fibrisolvens*, but also to *trans*-9,*trans*-11 CLA, although in considerably smaller amounts (Wallace et al., 2007), which is—just as *cis*-9,*trans*-11 CLA—further reduced to predominantly *trans*-11 18:1 (McIntosh et al., 2009; McKain et al., 2010). When incubated with 18:3n-3, *B. fibrisolvens* produces *cis*-9,*trans*-11,*cis*-15 CLnA, which is further reduced to *trans*-11,*cis*-15 18:2 (Kepler and Tove, 1967; Fukuda et al., 2009). Some *B. fibrisolvens* strains further convert this 18:2 isomer to *trans*-11 18:1, whereas *trans*-11,*cis*-15 18:2 is the end product of other strains (Fukuda et al., 2009). This suggests that biohydrogenation of 18:2n-6 and 18:3n-3 is strain specific, as observed by others (Gorissen et al., 2010; Hennessy et al., 2012; Hussain et al., 2016).

Among other *Butyrivibrio* species, *B. hungatei* is another species capable of producing *trans*-11 18:1 from 18:2n-6 (Maia et al., 2007; Paillard et al., 2007), whereas *B. proteoclasticus* (previously named *Clostridium proteoclasticum*, Moon et al., 2008) produces *trans*-11 18:1, but also further metabolizes this isomer to 18:0 (Maia et al., 2007; Wallace et al., 2007; McKain et al., 2010).

Several in vivo studies investigating the association between FA proportions and relative abundances of bacterial species found no correlation between the

abundance of *Butyrivibrio* spp. and *trans*-11 intermediates in the rumen or at the entrance of the omasal canal (Zened et al., 2016; Zhu et al., 2016; Kairenius et al., 2018), suggesting that other species are involved in ruminal *trans*-11 formation. Indeed, in vitro experiments revealed other bacteria capable of producing *trans*-11 intermediates from 18:2n-6 or 18:3n-3. They were isolated from the rumen, from the human, rat, or mouse intestine, or from other sources such as cheese, and mainly belong to the genera *Bifidobacterium* (Gorissen et al., 2010; Hennessy et al., 2012; Park et al., 2012), *Clostridium* (Verhulst et al., 1985), *Enterococcus* (Kishino et al., 2002), *Eubacterium* (Kemp et al., 1975; Eyssen and Verhulst, 1984), *Lactobacillus* (Kishino et al., 2002; Alonso et al., 2003; Renes et al., 2017), *Pediococcus* (Kishino et al., 2002), *Propionibacterium* (Devillard et al., 2007; McIntosh et al., 2009; Hennessy et al., 2012), *Pseudobutyrvibrio* (Paillard et al., 2007), *Roseburia* (Devillard et al., 2007; McIntosh et al., 2009), *Ruminococcus* (Kemp et al., 1975; McIntosh et al., 2009), and *Sharpea* (Dewanckele et al., 2019c). However, ruminal abundance of those genera is generally lower as compared with the genus *Butyrivibrio*, with the exception of genus *Ruminococcus* (Henderson et al., 2015). Furthermore, *Butyrivibrio* spp. was suggested to isomerize 18:2n-6 to *cis*-9,*trans*-11 CLA more rapidly than any other bacterial species (Paillard et al., 2007; Shingfield et al., 2012; Hussain et al., 2016), although in most pure culture studies reported in Table 2, this could not be confirmed, as kinetic assessments were lacking. Furthermore, interexperiment comparisons of pure culture studies are challenging, due to diversity in growth media, which might affect the growth and biohydrogenating activity, as some bacteria might face suboptimal conditions. Additionally, Huws et al. (2011) found that *cis*-9,*trans*-11 CLA and *trans*-11 18:1 are linked with uncultured bacteria, phylogenetically classified as genera *Prevotella*, *Lachnospiraceae incertae sedis*, *Ruminococcus*, *Butyrivibrio*, *Pseudobutyrvibrio*, *Tannerella*, and *Anaerovorax* and unclassified *Bacteroidales*, *Clostridia*, *Clostridiales*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *Porphyromonadaceae*. Hence, it is probable that uncultured bacteria are involved in ruminal biohydrogenation.

Alternatively, the lack of correlation between *Butyrivibrio* spp. and *trans*-11 intermediates may be due to the use of DNA as microbial marker in these in vivo studies, whereas metabolic activity is expected to be better related to RNA concentrations. Furthermore, microbial DNA-metabolite or microbial RNA-metabolite correlations remain indicative as they might be indirect, and interpretations are impeded because biohydrogenation intermediates are unstable in the rumen (continuous production and conversion).

**Table 2.** Overview of intermediates and end products of biohydrogenation of 18-carbon fatty acids by specific bacterial species<sup>1</sup>

Species	Isolated from	RA <sup>2</sup> (%)	Substrate	Intermediates and end products <sup>3</sup>	Initial conc. (µg/mL)	Substrate disappearance	Reference <sup>4</sup>
<i>Bifidobacterium adolescentis</i> , <i>Bifidobacterium bifidum</i> , <i>Bifidobacterium breve</i> , <i>Bifidobacterium (pseudo)longum</i> <i>Butyrivibrio fibrisolvens</i>	Human intestine, nursing stool, blood, chicken feces, (bovine) rumen, or unknown Cow, sheep or goat rumen, or human feces	<0.1 3.4	18:2n-6 18:3n-3 18:2n-6	c9, t11 CLA, t9, t11 CLA, OH-18:1 c9, t11, c15 CLnA, OH-18:2 c9, t11 CLA, t9, t11 CLA, t9, c11 CLA, t11 18:1, t9 18:1 c9, t11, c15 CLnA, t11, c15 18:2, t11 18:1	40–500 40–557 40–701 40–696	12–100% after 0–72 h 7–99% after 24–72 h 85–100% after 2–24 h 98–100% after 2–24 h	9, 14, 15, 17, 21 15, 17, 18, 21 1, 2, 9, 10, 12, 13, 14, 20, 21 2, 13, 20, 21
<i>Butyrivibrio hungatei</i> <i>Butyrivibrio proteoclasticus</i>	Sheep rumen Cow or sheep rumen	3.4 3.4	c9, t11 CLA t9, t11 CLA t10, c12 CLA 18:2n-6 18:3n-3	t11 18:1 t10 18:1, t12 18:1, c12 18:1 t11 18:1 c9, t11 CLA, t11 18:1, 18:0 c9, t11, c15 CLnA, t11, c15 18:2, t13/14 18:1, t15 18:1, c15 18:1	27 33 18 50 40–50 40	99% after 24 h 33% after 24 h 92% after 24 h Unknown 98% after 24 h 99–100% after 2–24 h	16 16 16 10, 11 10, 11, 21 20, 21
<i>Clostridium aminophilum</i> , <i>Clostridium bifementans</i> , <i>Clostridium sporogenes</i> <i>Enterococcus faecium</i> <i>Eubacterium lentum</i> or undefined	Rumen or mouse intestine Unknown Sheep rumen or rat feces	0.7 0.1 <0.1	t9, t11 CLA t11 18:1, t10 18:1 or c9 18:1 18:2n-6	18:0 18:0 c9, t11 CLA, c9 18:1, t11 18:1	33 41–48 20–50	19% after 30 h 40–93% after 30–72 h 100% after 48 h	16 16 5, 10
<i>Fusocillus babrahamensis</i> or undefined <i>Lactobacillus acidophilus</i> , <i>Lactobacillus brevis</i> , <i>Lactobacillus (para)casei</i> , <i>Lactobacillus pentosus</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus rhamnosus</i> <i>Megasphaera elsdenii</i>	Sheep rumen Human intestine, cheese, or unknown	<0.1 0.3	18:2n-6 18:3n-3 18:2n-6	c9, t11 CLA, t9, t11 CLA c9, t11 CLA, t11 18:1 c9, t11, c15 CLnA, t11, c15 18:2, t11 18:1, c11 18:1 c9, t11 CLA, t11 18:1, 18:0 c9, t11, c15 CLnA, t11, c15 18:2, c15 18:1 c9, t11 CLA, t9, t11 CLA, t10, c12 CLA	4,000 20–150 20 20 20 200–4,000	82% after 72 h 100% after 24 h Unknown Unknown Unknown 4–100% after 48–72 h	7 3, 4 3 3 3 7, 8, 19
<i>Mitsuokella multiacidus</i> <i>Pediococcus acidilactici</i> <i>Propionibacterium acnes</i> , <i>Propionibacterium freudenreichii</i> , <i>Propionibacterium shermanii</i>	Cow rumen Rumen Unknown Sheep rumen, acne lesion in human facial skin, dairy starter, cheese, or unknown	<0.1 <0.1 <0.1 <0.1	18:2n-6 18:3n-3 18:2n-6 18:2n-6 18:3n-3	t10, c12 CLA, Δ9,14–18:2, OH-18:1 OH-18:2 c9 18:1 c9, t11 CLA, t9, t11 CLA c9, t11 CLA, t9, t11 CLA, t10, c12 CLA c9, t11, c15 CLnA, t10, c12, c15 CLnA, Δ11,13,15–18:3 10-OH-18:0, 10-O-18:0	20–40 40 50 4,000 40–4,000 40–450 41	82–88% after 24 h 33–61% after 24 h Unknown 68% after 72 h 2–100% after 24–96 h 2–87% after 24–72 h 29–89% after 96 h	6, 21 21 10 7 7, 9, 12, 14, 17, 21 17, 21 16
<i>Roseburia hominis</i> , <i>Roseburia inulinivorans</i>	Human feces	0.1	t10 18:1 or c9 18:1 18:2n-6	c9, t11 CLA, t11 18:1	50–500	100% after 0–24 h	9, 14

Continued

**Table 2 (Continued).** Overview of intermediates and end products of biohydrogenation of 18-carbon fatty acids by specific bacterial species<sup>1</sup>

Species	Isolated from	RA <sup>2</sup> (%)	Substrate	Intermediates and end products <sup>3</sup>	Initial conc. (µg/mL)	Substrate disappearance	Reference <sup>4</sup>
<i>Ruminococcus albus</i>	Sheep rumen	3.6	18:2n-6	c9, t11 CLA, t11 18:1, t10 18:1	20	Unknown	3
			18:3n-3	c9, t11, c15 CLnA, t11, c15 18:2, t11 18:1, t10 18:1	20	Unknown	3
<i>Ruminococcus obeum</i>	Human feces	3.6	18:2n-6	c9, t11 CLA, t11 18:1	500	100% after 24 h	14
<i>Sharpea azabuensis</i>	Calf rumen or horse feces	0.2	18:2n-6	c9, t11 CLA, t11 18:1	40	100% after 8 h	20
			18:3n-3	c9, t11, c15 CLnA, t11, c15 18:2	40	100% after 8 h	20
<i>Streptococcus bovis</i> , <i>Streptococcus equinus</i> , <i>Streptococcus gallolyticus</i>	Sheep rumen, cow dung, or koala feces	0.5	18:2n-6	Δ9,14-18:2, OH-18:1	40–50	90% after 24 h	10, 21
			18:3n-3	OH-18:2	40	22–96% after 24 h	21

<sup>1</sup>Differences between studies in initial substrate concentration (initial conc.) and time of incubation (shown together with substrate disappearance) were large. The range is shown for those parameters.

<sup>2</sup>RA = the mean relative abundance in the rumen on genus level, based on Henderson et al., 2015.

<sup>3</sup>t = *trans*; c = *cis*; CLnA = conjugated linolenic acid.

<sup>4</sup>1 = Kepler et al., 1966; 2 = Kepler and Tove, 1967; 3 = Kemp et al., 1975; 4 = Eysen and Verhulst, 1984; 5 = Verhulst et al., 1985; 6 = Kim et al., 2002; 7 = Kishino et al., 2002; 8 = Alonso et al., 2003; 9 = Devillard et al., 2007; 10 = Maia et al., 2007; 11 = Paillard et al., 2007; 12 = Wallace et al., 2007; 13 = Fukuda et al., 2009; 14 = McIntosh et al., 2009; 15 = Gorissen et al., 2010; 16 = McKain et al., 2010; 17 = Hennessy et al., 2012; 18 = Park et al., 2012; 19 = Renes et al., 2017; 20 = Dewanckele et al., 2019c; 21 = L. Dewanckele, J. Jeyanathan, B. Vlaeminck, V. Fievez, Ghent University, Ghent, Belgium, unpublished data.

### Bacteria Involved in the *Trans*-10 Pathway

Bacterial species involved in ruminal *trans*-10 formation are not well known. Kemp et al. (1975) observed formation of *trans*-10 18:1 from 18:2n-6 and 18:3n-3 by *Ruminococcus albus*. Interestingly, ruminal abundance of the genus *Ruminococcus* is comparable with the abundance of the genus *Butyrivibrio* (Henderson et al., 2015). However, the relative abundance of this genus was not correlated with the rumen proportion of *trans*-10 18:1 in the study of Dewanckele et al. (2018), and moreover, *Ruminococcus* abundance was lower in situations with greater *trans*-10 accumulation in the experiment of Dewanckele et al. (2019b). Unfortunately, *Ruminococcus* was not identified on species level in either study, which impairs appropriate strain selection for pure culture studies. To our knowledge, no other studies reported 18:2n-6 or 18:3n-3 metabolism by *R. albus* since 1975.

Milk fat depression or situations with greater *trans*-10 accumulation are often associated with increased ruminal abundance of *Megasphaera elsdenii* (Weimer et al., 2010; Mohammed et al., 2012; Dewanckele et al., 2018), which has been suggested to have a potential role in the production of *trans*-10 intermediates from 18:2n-6 or 18:3n-3. Indeed, Kim et al. (2002) found 2 strains of ruminal *M. elsdenii* converting 18:2n-6 to *trans*-10, *cis*-12 CLA. In contrast, neither of the 2 *M. elsdenii* strains in the study of Maia et al. (2007) formed *trans*-10, *cis*-12 CLA, although this study included the T81 strain tested before by Kim et al. (2002). This concurs with observations in our laboratory using 14 different rumen strains of *M. elsdenii* (Dewanckele et al., 2019a).

In vitro experiments with *Lactobacillus* spp., isolated from cheese (Renes et al., 2017) or from the human intestine (Alonso et al., 2003), revealed *trans*-10, *cis*-12 CLA formation from 18:2n-6 by different species of this genus. Furthermore, the ruminal abundance of this genus correlated positively with *trans*-10 18:1 in the study of Dewanckele et al. (2018). Additionally, *Bifidobacterium pseudolongum* isolated from the rumen produced *trans*-10, *cis*-12 CLA in the in vitro study of Jaglan et al. (2019). Nevertheless, the contribution of both species to the in vivo ruminal *trans*-10 formation remains to be elucidated.

Devillard et al. (2007) and McIntosh et al. (2009) observed formation of *trans*-10, *cis*-12 CLA from 18:2n-6 by *Propionibacterium freudenreichii*, a bacterial species isolated from the human intestine. In line with this, Wallace et al. (2007) found that *Propionibacterium acnes*, which was isolated from sheep rumen, converts 18:2n-6 to *trans*-10, *cis*-12 CLA as an end product (McKain et al., 2010; Dewanckele et al., 2017). Nevertheless, *B. fibrisolvens* reduces *trans*-10, *cis*-12 CLA to *trans*-10



18:1 in vitro (Kepler et al., 1966; McKain et al., 2010), which could explain the further reduction of *trans*-10,*cis*-12 CLA in vivo (Shingfield and Wallace, 2014). *Propionibacterium acnes* also converts 18:3n-3 to various biohydrogenation intermediates, but the formation of *trans*-10,*cis*-12,*cis*-15 CLnA is equivocal: although this isomer was not found during in vitro studies of Maia et al. (2016), the same strain of *P. acnes* isomerized part of 18:3n-3 to *trans*-10,*cis*-12,*cis*-15 CLnA in our laboratory (Dewanckele et al., 2019a). In both experiments, no 18:2 or 18:1 FA formation was observed. Those observations suggest that *P. acnes* might be involved in ruminal formation of *trans*-10 intermediates. However, *P. acnes* is one of the less numerous ruminal species (Shingfield et al., 2012; Kairenius et al., 2018), questioning its predominant role in ruminal *trans*-10 formation in vivo. Nevertheless, substrate affinity, metabolic kinetics, as well as environmental conditions are other potential factors determining the competitive contribution of this species to the conversion of 18:2n-6 in the rumen.

As suggested for bacteria that produce *trans*-11, as yet uncultured bacteria potentially might be involved in ruminal *trans*-10 formation. Correlation analysis based on ruminal bacterial populations and milk (Pitta et al., 2018; Dewanckele et al., 2019b) or rumen FA profiles (Zened et al., 2016; Dewanckele et al., 2018) indicated the following genera or species to be positively related to *trans*-10-biohydrogenation intermediates: *Acidaminococcus* spp., *Bulleidia* spp., *Bifidobacterium* spp., *Carnobacterium* spp., *Desulfovibrio* spp., *Dialister* spp., *Eubacterium* spp., *Olsenella* spp., *Sharpea* spp., and *Syntrophococcus* spp. and unclassified *Coriobacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae*. However, in vitro studies using pure cultures are needed to ascertain their capacity to produce *trans*-10 intermediates from 18:2n-6 or 18:3n-3. For the unclassified taxa, this is impossible. Furthermore, genera, species and strain specificity of PUFA biohydrogenation (Gorissen et al., 2010; Hennessy et al., 2012; Hussain et al., 2016) complicates such studies. Isolation of bacterial strains from the rumen of animals suffering from MFD using a dilution-to-enrichment (or even “extinction”) approach with 18:2n-6 or 18:3n-3 as a selection component could potentially reveal other *trans*-10 producing bacteria. This approach has been used before to isolate previously uncultured ruminal bacteria (Kenters et al., 2011). Briefly, rumen fluid is serially diluted to produce cultures with decreasing amounts of inoculum, whereas the concentration of the selection component, such as 18:2n-6 or 18:3n-3 in case of biohydrogenating bacteria, is kept constant. Dilution of the inoculum will result in a less diverse culture, presumably shifting toward relatively more bacteria able to convert 18:2n-6 or

18:3n-3. A limitation of this approach is the need to use PUFA as the selection component. Indeed, PUFA are toxic to microbes, and biohydrogenating microbes do not generate energy through this process. Hence, in contrast to isolation (lactate-utilizing bacteria using lactate as a selection component), application of the dilution-to-extinction approach represents a “survival of the fittest” concept in the context of biohydrogenation.

Contrasting results of studies using the same bacterial species or strains are another complication associated with the search for bacteria involved in *trans*-10 biohydrogenation pathways. This might indicate that not only the species or strain itself is important, but that also specific conditions, which are apparently still unknown, are needed to produce *trans*-10 intermediates. Possibly, these specific conditions were (accidentally) fulfilled in experiments where *trans*-10 production was observed with a certain strain (*P. acnes* by Wallace et al., 2007 and Dewanckele et al., 2019a; *M. elsdenii* by Kim et al., 2002), whereas this was not the case in other studies using the same strain (*P. acnes* by Maia et al., 2016; *M. elsdenii* by Maia et al., 2007 and Dewanckele et al., 2019a). In the study of Jaglan et al. (2019), MRS broth was used as growth medium, which is a selective medium for *Bifidobacteria* (Sigma-Aldrich, Diegem, Belgium). This medium might have been more stimulating for *B. pseudolongum* to produce *trans*-10,*cis*-12 CLA compared with the medium used in our laboratory (as described in Dewanckele et al., 2019c). However, the medium used in our laboratory was more closely related to rumen conditions, which is more relevant in studies related to rumen metabolism. Similarly, a low pH or supplementation of DHA to the medium negatively affected the rate of biohydrogenation in the study of Dewanckele et al. (2017), which also demonstrated a role of environmental conditions. Other in vitro experiments in our laboratory suggested that conditions inducing MFD (in that case, supplementation of DHA) reduced the formation rate of *trans*-11 intermediates, rather than increased the formation rate of *trans*-10 intermediates [L. P. Thanh (Can Tho University, Can Tho, Vietnam), B. Vlaeminck, and V. Fievez, unpublished data]. The results of Dewanckele et al. (2017) even showed a reduction in the formation rate of both *trans*-11 and *trans*-10 intermediates by MFD-inducing conditions (in that case, low pH or supplementation of DHA), although this reduction seemed less pronounced for *trans*-10 intermediates. Possibly, other circumstances or metabolites, besides lactate or marine lipid supplementation or the creation of a low pH environment, might influence the biohydrogenating activity of certain species, and perhaps alter their pathway.

Overload of the *trans*-11 pathway has been another hypothesis for the shift toward the formation of *trans*-10 intermediates (Vlaeminck et al., 2014). This implies that *trans*-10-producing bacteria start converting 18:2n-6 or 18:3n-3 only when *trans*-11 intermediates accumulate, which is in line with the lag time of *trans*-10 appearance when feeding high starch-low fiber diets or supplementing marine products. For example, switching from a control diet to a diet low in fiber and high in starch first induced an increase in *cis*-9,*trans*-11 CLA and *trans*-11 18:1 in the experiment of Rico and Harvatine (2013), which was presumably caused by a limited capacity of the final steps of the normal biohydrogenation pathway. After 5 d, those *trans*-11 intermediates declined, whereas *trans*-10,*cis*-12 CLA and *trans*-10 18:1 increased. Furthermore, supplementation of marine lipids always seems to inhibit the final biohydrogenation step to 18:0, resulting in the accumulation of *trans*-11 18:1, whereas an increase in *trans*-10 18:1 is not always observed (Boeckaert et al., 2007; Zhao et al., 2016; Dewanckele et al., 2018). Hence, it was hypothesized that a certain *trans*-11 threshold could be a prerequisite for *trans*-10 formation, which was assessed by Vlaeminck et al. (2014). However, based on their in vitro results, *trans*-11 18:1 accumulation was excluded as a major factor explaining *trans*-10 18:1 production.

Taking all these findings into consideration, it seems unlikely that the abundance of specific bacterial species or strains capable of producing *trans*-10 intermediates are determining the occurrence of a *trans*-10 shift, and other indirect effects might play a crucial role.

### Bacteria Associated with FA Hydration

Some bacteria produce hydroxy FA from 18:3n-3, 18:2n-6, or *cis*-9 18:1, such as strains within the genera *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, and *Staphylococcus* (Hudson et al., 1998, 2000; Maia et al., 2007), as well as the genera *Megasphaera* and *Bifidobacterium* (Dewanckele et al., 2019a). According to Jenkins et al. (2006), hydroxy FA formed upon UFA hydration might be further oxidized to oxo FA. Several recent in vivo studies observed a positive association between MFD and the milk fat proportion of oxo-18:0, particularly of 10-oxo-18:0 (Bernard et al., 2015; Kairenius et al., 2015); therefore, it is worth investigating this ruminal metabolic pathway. In vivo or in vitro studies with cows (Torral et al., 2016a; Carreño et al., 2019) or sheep (Carreño et al., 2019) suggested the potential involvement of species within the *Veillonellaceae* family, such as *Quinella ovalis* and unclassified *Veillonellaceae*, in the hydration of UFA to 10-oxo-18:0. Nevertheless, further in vitro studies using pure cultures are needed to confirm this hypothesis.

### POTENTIAL STRATEGIES TO AVOID MFD

Given the questions regarding the identification of FA and microbes causing or associated with MFD, it might be questioned if the underlying mechanisms of diet-induced MFD really need to be unraveled to develop dietary and management practices to avoid this situation. Although MFD may be used as a management tool in some specific situations in dairy cow husbandry (Bauman et al., 2011), more often it represents a negative condition. Therefore, strategies to avoid diet-induced MFD generally are of interest.

Indeed, some empirically developed strategies have shown some success in preventing or minimizing the risk of MFD. It is clear that an imbalanced rumen environment is the basis of diet-induced MFD. As such, avoiding imbalanced rumen conditions could minimize the risk. In this respect, Rico et al. (2014) investigated the effect of rumen digesta inoculation from non-MFD cows on the recovery from diet-induced MFD. Although no change in milk fat yield was observed, ruminal inoculation from non-MFD cows accelerated the rate of recovery of mammary de novo FA synthesis and normal ruminal biohydrogenation. Obviously, such strategy is not useful in practice. Yet, it demonstrated the possibility to improve MFD-recovery or to prevent MFD by rumen interventions. Supplements supporting a more resilient rumen environment might thus decrease the risk for MFD. Stabilization of ruminal fermentation has been suggested as a benefit of yeast culture supplementation (Williams et al., 1991). Supplementation of yeasts has been reported to increase the ruminal pH by decreasing the accumulation of lactate through stimulation of growth and metabolism of the lactate-utilizing bacteria *Selenomonas ruminantium* and *M. elsdenii* (Vohra et al., 2016). Furthermore, an in vitro study by Newbold et al. (1996) using 2 strains of *Saccharomyces cerevisiae* and their respiration-deficient mutants showed that yeast supplementation stimulates the cellulolytic bacterial populations, which include *Butyrivibrio* spp. (Shane et al., 1969). Longuski et al. (2009) investigated the effects of yeast culture supplementation on responses to a fermentable starch challenge and concluded that yeast culture supplementation may help prevent MFD during transition to a diet high in RFCH. More recently, Baldin et al. (2018) observed a decreased risk for MFD by the rumen modifier 2-hydroxy-4-methylthiobutanoate, a methionine analog (HMTBa). A meta-analysis performed by Zanton et al. (2014) described an increased milk fat yield with HMTBa supplementation, but it was inconclusive on the role of HMTBa in MFD circumstances. Baldin et al. (2018) evaluated the interaction between dietary risk factors, cow milk production level, and supplement-

tation of HMTBa. In their study, 16 high-producing and 14 low-producing dairy cows were fed diets with increasing risk of MFD. The increased risk of MFD was achieved by decreasing the dietary NDF content in combination with supplementation of soybean oil. Only the high-producing cows experienced MFD upon increasing MFD risk, which was not observed when HMTBa was included in the diet at 1 g/kg DM. This higher milk fat content and yield of high-producing cows supplemented with HMTBa was accompanied with a decreased milk fat concentration of *trans*-10 18:1 and an increased concentration of *trans*-11 18:1. This indicates a role of HMTBa in stabilizing rumen biohydrogenation and reducing the *trans*-11 to *trans*-10 shift.

However, the interaction of the rumen modifier (i.e., HMTBa) with dietary MFD risk factors and cow milk production level indicates diet-induced MFD is the result of numerous factors, including interanimal variation. The latter has been observed in dairy cows, goats, and ewes (Frutos et al., 2017; Baldin et al., 2018; Dewanckele et al., 2019b) and might be related to eating behavior, rumen microbial community, genetics, or a combination of these and other interacting factors. Indeed, Nasrollahi et al. (2017) observed a higher DMI and decreased sorting behavior, whereas Weimer et al. (2010) observed lower shifts in rumen bacterial community in MFD-tolerant animals. Potentially, selection toward MFD-tolerant animals might decrease the incidence of MFD on dairy farms in future. However, this might be associated with lower milk production levels, because high-productive animals seemed more vulnerable to MFD as compared with low-productive animals (Baldin et al., 2018). This shift to lower productive animals is not necessarily negative, because lower productive animals might be associated with higher fertility, less leg and metabolic problems, and increased longevity (Oltenacu and Broom, 2010), which is perceived advantageous in terms of animal welfare. This is regarded as more sustainable by the public and may therefore be economically beneficial (Oltenacu and Broom, 2010), provided that consumers are willing to pay more for sustainable milk (products) if production costs would increase. Nevertheless, in the experiment of Dewanckele et al. (2019b), the cows with the highest milk fat content and yield throughout the experiment also showed higher milk production. This might indicate that selection toward MFD-tolerant animals is not necessarily associated with lower milk and milk fat production levels and, more importantly, might result in increased feed efficiency.

Perhaps the fastest and easiest solution to diet-induced MFD is to not feed diets that could induce MFD. Lowering the dietary proportion of concentrates

and grains could decrease the incidence of diet-induced MFD on modern dairy farms. Moreover, this decreased use of human-edible grains in the dairy ration would increase the sustainability of dairy farms, as the feed-food competition and thus, the need for arable land would be diminished (Van Zanten et al., 2018). However, it should be noted that concentrates in the dairy industry also contain by-products next to cereal grains, which are not human-edible, such as sugar beet pulp and brewer's meal. Some of these by-products contain plant secondary compounds, such as tannins, which might positively modulate ruminal biohydrogenation (including, in some cases, reductions in *trans*-10 18:1; Khiaosa-ard et al., 2009; Vasta et al., 2010). Above this, lowering the dietary proportion of grains might have a negative effect on the emission of methane. Cattle fed forage diets produce a greater proportion of total VFA as acetate, and thus more hydrogen is available for methanogenesis (Janssen, 2010). As such, decreasing the proportion of grains might be sustainable from a land-use perspective, but might not be so in terms of the climate effect, although the overall carbon footprint, not just methane, should be considered. The composition of concentrates, in particular the proportion of by-products and the inclusion of soybean meal, largely determines the latter (Hörtenhuber et al., 2011). Furthermore, grains are necessary in early lactation to supply glucogenic precursors, which are a determining factor for maintaining a balanced liver metabolism. As such, feeding less concentrate might result in a higher prevalence of (subclinical) ketosis. Indeed, increasing the forage:grain ratio of the diet from lactating Holstein cows from 38.2:61.8 to 98.2:1.8 increased the BHB concentration in blood 3-fold during the first 4 wk of lactation in the study of Dhiman et al. (1991). In this respect, selection toward more efficient fiber-digesting cows could be useful.

Feeding less grains and concentrate might also decrease milk yield. This might result in an economic loss for the dairy farmer if the production efficiency decreases, but it might also complicate feeding an increasing population. According to Willet et al. (2019), a healthy and sustainable reference diet consists of 250 g/d of whole milk or derivative equivalents, such as cheese. This reference amount of 250 g/d is based on extensive literature on foods, dietary patterns, and health outcomes. The United Nations estimates that the world's population will grow from 7.7 to 10.5 billion between 2019 and 2069 (United Nations, 2017). Using 250 g/d as reference, the required milk production to feed the world's population will increase from 703 to 958 billion kg/yr. In 2018, nearly 827 billion kg of milk was produced worldwide (FAO, 2018). As such,



required milk production is expected to increase 16% in the next 50 years. Obviously, the consumption of dairy products is skewed worldwide. Western Europe, for example, consumes far above the reference level of 250 g/d (on average 820 g of milk equivalents/d; FAO, 2018), compared with less than 80 g/d (and even sometimes as little as 27 g/d) in some African and Asian countries, which is far below the level of a healthy and sustainable reference diet. Even if milk consumption in Western Europe would decrease by 70% (from 820 to 250 g/d), which is unlikely, an increased milk production is expected, because in developing countries, the demand for milk (products) increases as the economy develops. An illustration of this is the predicted 3.2-fold increase in demand in China by 2050 compared with the production level in 2010 (Bai et al., 2018).

As such, a plethora of strategies to optimize dairy production is of interest. With regards to reduced MFD risk, these might include supplementation of rumen stabilizers (e.g., yeast, HMTBa), selection toward more tolerant animals, tailored management of cows at risk for MFD, selection toward more efficient fiber digesting cows and feeding less grains and concentrate.

## CONCLUSIONS

Although diet-induced MFD seems to be a multi-etiological syndrome, in the vast majority of MFD cases, a shift occurs in the rumen biohydrogenation pathway from the formation of mainly *trans*-11 intermediates toward a greater accumulation of *trans*-10 intermediates (i.e., *trans*-10, *cis*-12 CLA, *trans*-10 18:1 and *trans*-10, *cis*-15 18:2), which also is referred to as the *trans*-11 to *trans*-10 shift. Nevertheless, further research is needed to confirm the direct role of *trans*-10 18:1 and *trans*-10, *cis*-15 18:2 in MFD. The microbial etiology of this *trans*-11 to *trans*-10 shift is not well understood. *Butyrivibrio* spp. are still the best known *trans*-11 producing ruminal bacteria; however, the ruminal bacteria responsible for *trans*-10 formation are unclear. Inconsistent results in the literature suggest that other as yet uncultured bacteria might be involved or that specific conditions are needed to produce *trans*-10 isomers. Further research using functional metagenomics and metabolomics approaches might provide further insight into the microbiology of diet-induced changes in bacterial biohydrogenation within the rumen. Nevertheless, it seems that unraveling the microbial mechanisms of diet-induced MFD is challenging. Potential strategies to avoid MFD are supplementation of rumen stabilizers, selection toward more tolerant animals, tailored management of cows at risk for MFD, selection toward more efficient fiber digesting cows, and feeding less concentrates and grains.

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