EFFECTS OF DIETARY FACTORS AND RUMEN PH ON RUMEN BIOHYDROGENATION PATHWAYS AND RISK OF MILK FAT DEPRESSION

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ABSTRACT

EFFECTS OF DIETARY FACTORS AND RUMEN PH ON RUMEN BIOHYDROGENATION PATHWAYS AND RISK OF MILK FAT DEPRESSION

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Yield of milk components continues to be the principal driver of variation in producer milk payments. Therefore, diet-induced milk fat depression negatively impacts financial income of dairy farmers. Our overall objective is to determine the effects of dietary factors and rumen pH and their interactions on biohydrogenation pathways and the formation of biohydrogenation intermediates (*e.g.* *t*10, *c*12 conjugated linoleic acid, [CLA]) that limit fat synthesis in the mammary gland. By using an in vitro batch culture system in the first three experiments, we determined the effects of common dietary factors (dietary unsaturated fatty acid concentration, starch content and starch fermentability, and *Saccharomyces cerevisiae* fermentation product) and culture pH on biohydrogenation pathways, as well as their interactions. In all three experiments, culture pH had the greatest influence on biohydrogenation pathways, with low culture pH increasing the formation of *t*10*, c*12 CLA in vitro. In the first experiment, low culture pH and increasing concentration of corn oil increased the formation of *t*10, *c*12 CLA. Increasing corn oil concentration at low culture pH increased *t*10, *c*12 CLA concentration. In the second experiment, low culture pH, combined with highly fermentable starch (high moisture corn), increased *t*10, *c*12 CLA concentration. Although starch fermentability did not affect *t*10, *c*12 CLA overall, high starch content provided by high moisture corn increased *t*10, *c*12 CLA concentration at low culture pH. In the third experiment, highly fermentable starch (high

moisture corn) at low culture pH increased *t*10, *c*12 CLA concentration. Rumen fluid collected from cows supplemented with *Saccharomyces cerevisiae* fermentation product decreased *t*10, *c*12 CLA concentration, especially when combined with high moisture corn at low culture pH. The fourth experiment was an in vivo study, which determined the effect of production level on severity of diet-induced milk fat depression and biohydrogenation pathways for mid- and late lactation cows. A milk fat depression-inducing diet decreased milk fat content and fat yield, and increased *t*10, *c*12 CLA concentration in milk. Higher producing cows were at higher risk for diet-induced milk fat depression, exhibiting greater reductions in milk fat content and yield and a greater increase in milk *t*10, *c*12 CLA concentration than lower producing cows. Cows fed a milk fat depression-inducing diet had a lower mean rumen pH and greater rumen pool of *t*10, *c*12 CLA than cows fed a control diet. Dietary factors interacted with rumen pH to influence biohydrogenation pathways and *t*10, *c*12 CLA concentration, and production level also impacted cow response to diet-induced milk fat depression. Further work is required to clarify interactions between dietary factors and rumen pH and their effects on rumen bacterial populations. Mechanisms behind the interaction between production level and diet induced-milk fat depression are still unclear and should be examined.

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KEY TO ABBREVIATIONS

AOAC	Association of Official Agricultural Chemists
A-RF	Saccharomyces cerevisiae fermentation product adapted rumen fluid
BCS	body condition score
BH	biohydrogenation
BW	body weight
Ca	calcium
CLA	conjugated linoleic acid
CO	corn oil treatment
CON	control treatment
CP	crude protein
CpH	culture pH treatment
CV	coefficient of variation
d	day (s)
DC	dry ground corn treatment
DE	digestible energy
DIM	days in milk
DM	dry matter
DMI	dry matter intake
ECM	energy-corrected milk
FA	fatty acid(s)
FAME	fatty acid methyl ester(s)

FCM	fat-corrected milk
FID	flame-ionization detector
g	gram(s)
GLC	gas-liquid chromatography
h	hour(s)
HMC	high moisture corn treatment
i.d.	internal diameter
kg	kilogram(s)
LA	linoleic acid
M	moles per liter
MBW	metabolic body weight
Mcal	megacalories
ME	metabolized energy
MFD	milk fat depression
MFDI	milk fat depression-inducing treatment
min	minutes(s)
mL	milliliter(s)
mm	millimeter(s)
μm	micrometer(s)
MUFA	monounsaturated FA
n	number
N_2	nitrogen gas
NDF	neutral detergent fiber

NE	net energy
NEFA	non-esterified fatty acid
NE_L	net energy for lactation
NRC	National Research Council
OBCFA	odd- and branched-chain fatty acid
pMY	preliminary milk yield
PUFA	poly-unsaturated fatty acid
R^2	coefficient of determination
RUFAL	rumen unsaturated fatty acid load
SAS	Statistical Analysis System
SC	starch content
SCFP	Saccharomyces cerevisiae fermentation product
SE	standard error
SEM	standard error of the mean
SF	starch fermentability
SFA	saturated fatty acid
TAG	triglyceride
TMR	total mixed ration
Trt	P-value associated with the treatment effect
UFA	unsaturated fatty acid
U-RF	Saccharomyces cerevisiae fermentation product unadapted rumen fluid
VFA	volatile fatty acid(s)

INTRODUCTION

Based on the Multiple Component Pricing system for Federal Milk Marketing Orders, the yield of milk components is the principal driver of variation in producer milk price. Compared to other milk components, fat is typically the most easily manipulated by nutrition and management. Some dietary conditions can cause decreases in milk fat yield, including diets containing large amount of readily fermentable carbohydrates and low forage content and diets supplemented with highly unsaturated oil (Bauman and Griinari, 2001). Diet-induced milk fat depression (MFD) is defined as a reduction of up to 50% in milk fat yield with no change in the yields of milk and other milk components. Consequently, MFD can cause approximately 2.5% loss of milk income for dairy farmers, based on the Mideast Federal Milk Marketing order price in November 2016.

Current evidence indicates that the biohydrogenation (BH) theory can explain most instances of MFD (Bauman and Griinari, 2001). Rumen bacteria biohydrogenate dietary unsaturated fatty acids (FA) and produce many different intermediates (Shingfield and Wallace, 2014). Specific FA intermediates (*e.g.* *t*10, *c*12 CLA) produced by altered biohydrogenation pathways can leave the rumen, be absorbed, inhibit milk fat synthesis in mammary gland and cause MFD. The occurrence of MFD requires two conditions, changes in rumen environment or rumen bacteria population and the presence of dietary unsaturated FA (Bauman et al., 2011). Through affecting these two conditions, some dietary factors can cause shifts in rumen BH pathways and alter the outflow of BH intermediates-associated with MFD.

Therefore, the key to both avoiding and troubleshooting MFD is understanding the complex relationships among diet, rumen BH and milk fat synthesis. Although we have a good understanding of the inhibitory effects of MFD-associated intermediates on the mammary gland,

there is limited information regarding dietary and rumen factors that promote the formation of those intermediates in the rumen. A limited number of in vitro studies have reported effects of rumen pH and individual dietary factors on BH. However, most studies did not take into account the interaction between dietary and rumen factors, which typically impact the BH in rumen and increase the risk for MFD.

To understand the complex relationships among diet, rumen BH, and MFD, we investigated the effects of dietary factors and rumen pH and their interactions on BH pathways in a series of in vitro studies, and the effect of production level on severity of diet-induced MFD and BH pathways in vivo. Our long-term goal is to develop effective feeding strategies to prevent MFD on dairy farms and promote maximal milk fat yield. The overall objective was to determine the interactions between dietary factors (dietary unsaturated FA concentration, starch content and starch fermentability, and *Saccharomyces cerevisiae* fermentation product) and rumen pH on BH and risk of MFD and the variation in cow responses to diet-induced MFD. Our central hypothesis was that, of the tested MFD risk factors, pH alteration (within physiological range) would have the greatest impact on BH pathway and milk fat synthesis, with increased formation of MFD-associated BH intermediates at low pH, and the negative effects of other factors on BH pathway would be enhanced by low pH.

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CHAPTER 1

LITERATURE REVIEW

Dairy cow dietary lipids

Sources of dietary lipid in dairy cow diets include concentrates, forages, oilseed products, and commercially available fat supplements. Generally, cereal grains and corn silages contain high concentrations of linoleic acid (LA, *c*9, *c*12 18:2), while α-linolenic acid (LNA, *c*9, *c*12, *c*15 18:3) is the most abundant FA in grass and legume forage sources. The major fatty acids (FA) in oilseeds include oleic oil (OL, *c*9 18:1), LA, and LNA (Shingfield and Wallace, 2014). Commercial fat supplements are often added to diets to increase dietary energy. The major FA in these supplements are typically palmitic acid (PA, 16:0), OL, and stearic acid (SA, 18:0; Weiss et al., 2011).

Lipid metabolism in the rumen

Dietary FA composition has less influence on the milk FA composition of ruminant animals than monogastric animals. Although major dietary FA are UFA (unsaturated FA), the FA reaching the intestine are mostly saturated due to the lipid metabolism in the rumen (Harfoot and Hazlewood, 1997). Rumen bacteria modify dietary lipids extensively, impacting the profile of FA available for intestinal absorption and tissue utilization (Palmquist et al., 2005). Two major modification processes that occur in the rumen include: 1) hydrolysis of ester linkages in lipids, which releases free FA; 2) subsequent biohydrogenation (BH) of UFA, which reduces the toxicity of UFA to rumen bacteria (Figure 1.1).

Hydrolysis

Most dietary lipids are in the forms of triglycerides (TAG), glycolipids, or phospholipids. After consumption and mastication, lipids are rapidly hydrolyzed. Rumen bacteria, rather than

protozoa and fungi, are the main microbes that perform hydrolysis in the rumen (Harfoot and Hazlewood, 1997). Microbial lipases release FA from their glycerol backbone through hydrolysis (Jenkins, 1993). Among all rumen bacteria, *Anaeovibrio lipolytica* is the most active and well-known bacteria that produces extracellular lipase and cell-bound esterase in order to hydrolyze triglycerides (Harfoot, 1997). *Butyrivibrio* spp. has also been shown to produce phospholipase A, phospholipase C, lysophospholipase, and phosphodiesterase (Harfoot and Hazlewood, 1997). Endogenous galactolipases and phospholipase in forage plant tissues can remain active for hours after ingestion and may also contribute to hydrolysis in the rumen (Lee et al., 2006; Van Ranst et al., 2009). However, the proportion of lipid hydrolyzed by plant-sourced galactolipases and phospholipase is not clear.

Biohydrogenation

Following hydrolysis, rumen bacteria biohydrogenate UFA to form saturated FA through isomerisation and hydrogenation, and produce many different intermediate ((Harfoot and Hazlewood, 1997; Shingfield and Wallace, 2014). Figure 1.2 shows the dominant BH pathway and the alternative pathway of LA. Several bacteria species are indentified and play important roles (McKain et al., 2010). In general, *Butyrivibrio fibrisolvens* isomerizes LA to rumenic acid (*c*9, *t*11 CLA; McKain et al., 2010). However, under specific dietary and rumen conditions, LA is isomerized to form *t*10, *c*12 CLA by *Megasphaera elsdenii* or *Propionibacterium acnes* (Kim et al., 2002; Wallace et al., 2007; Bauman et al., 2011). *Butyrivibrio fibrisolvens* hydrolyzes *c*9, *t*11 CLA and *t*10, *c*12 CLA to form vaccenic acid (*t*11 18:1) and *t*10 18:1, respectively. Finally, the *trans* 18:1 intermediates are hydrolyzed by *Butyrivibrio proteoclasticus* to form SA (McKain et al., 2010). The primary dietary UFA sources for BH are LA and LNA, and the rates of rumen BH for these FA range from 70-95% and 85-100%, respectively (Jenkins et al., 2008). Therefore,

SA, rather than UFA, is the predominant FA available for absorption by the dairy cow in typical feeding situations (Bauman and Lock, 2006). However, some BH intermediates and dietary UFA escape the rumen and are available for absorption in the small intestine along with SA (Figure 1.1). Absorbed FA are packaged into chylomicrons, transported in circulation, and subsequently become available to the mammary gland for milk fat synthesis.

Role of other rumen microbes

Protozoa account for approximately half of the rumen microbial biomass and contain high concentrations of BH intermediates including *c*9, *t*11 CLA and *t*11 18:1 (Devillard et al., 2006). Rather than directly participating in lipid metabolism, protozoa are considered to facilitate the escape of BH intermediates from the rumen, and therefore, increase the amount of UFA available for lower gut absorption (Or-Rashid et al., 2007). Although early studies suggested that protozoa could play an important role in BH (Wright, 1959 and 1960), only the bacteria that are engulfed by protozoa exhibit the enzyme activity needed for BH, and are therefore more likely to be responsible for BH (Dawson and Kemp, 1969; Harfoot and Hazlewood, 1997). Mixed rumen fungi have been shown to be capable of biohydrogenating LA to form *c*9, *t*11 CLA and *t*11 18:1. However, this activity is minor compared with that of *Butyrivibrio fibrisolvens* (Nam and Garnsworthy, 2007).

Biosynthesis of bacterial FA

Besides incorporating dietary FA, rumen bacteria are able to synthesize FA de novo. Odd- and branched-chain FA (OBCFA) in milk primarily originate from rumen bacterial membrane lipids, and therefore milk OBCFA can be used as a tool to predict rumen bacteria populations and rumen fermentation (Fievez et al., 2012). The major OBCFA include *iso*14:0, 15:0, *iso* 15:0, *anteiso* 15:0, *iso* 16:0, 17:0, and *iso* 17:0, *anteiso* 17:0 (Fievez et al., 2003b).

Cellulolytic bacteria contain higher proportions of even and odd- *iso* FA, in contrast to the amylolytic bacteria, which are more enriched by *anteiso* and linear odd-chain FA (Vlaeminck et al., 2006). Dietary factors affect the composition and quantity of OBCFA. Decreasing the dietary forage to concentrate ratio will decrease the ratio of *iso* FA to *anteiso* and linear odd-chain FA due to resulting decreased cellulolytic bacteria and increased amylolytic bacteria populations (Vlaeminck et al., 2006). Diets supplemented with LA and LNA result in low concentrations of OBCFA in milk (Collomb et al., 2004; Rego et al., 2005).

Milk fat synthesis and milk fat depression

Fat is the most energy dense component in whole milk and is responsible for many of the physical properties, organoleptic characteristics and manufacturing qualities of dairy products. Milk FA originate from two sources: < 16 carbon FA are synthesized de novo in the mammary gland and > 16 carbon FA are extracted from plasma as preformed FA. The mixed FA (16-carbon FA) can be derived from either de novo or preformed sources. Acetate and β-hydroxybutyrate, formed by rumen fermentation of carbohydrates, represent the major carbon sources for FA de novo synthesis in the mammary gland (Bauman and Griinari, 2003). In plasma, FA absorbed from the intestine are transported in lipoproteins and FA mobilized from body tissues are transported as nonesterified FA (Bauman and Griinari, 2003). Unless dairy cows experience negative energy balance, FA in lipoproteins are the major preformed FA utilized by the mammary gland to synthesize milk fat (Bauman and Griinari, 2001). Diets supplemented with saturated long chain FA (PA or SA) have been shown to increase yields of corresponding FA fed (Piantoni et al., 2013; Rico et al., 2014a; Piantoni et al., 2015).

By assessing the value of milk components at the farm level, an economic analysis by St-Pierre (2011) showed that 5% increases in yields of fat, protein, and milk would increase net

income of dairy farms by 13, 15, and 3%, respectively. Although increasing milk protein yield an equivalent amount would typically result in greater profit than increasing milk fat yield, milk fat is more easily manipulated by nutrition and management. For example, diets supplemented with long chain saturated FA are known to increase yield of milk fat (Piantoni et al., 2013; Rico et al., 2014a; Piantoni et al., 2015). On the other hand, other dietary conditions can cause reductions in milk fat yield, including diets supplemented with highly unsaturated oils and diets containing large amounts of readily fermentable carbohydrates and low forage content (Bauman and Griinari, 2001). Diet-induced milk fat depression (MFD) is defined by a reduction in milk fat yield of up to 50% without changes in other milk components and milk yield (Bauman and Grrinari et al., 2001). MFD can cause significant financial losses for farmers, when farmers are primarily paid based on yield of milk components. For example, keeping other milk component yields constant, a subtle decrease in fat concentration from 3.7 to 3.5% could result in $102,500/year income loss (2.5% loss of income) on a farm with 1,000 lactating dairy cows (based on the Mideast Federal Milk Marketing order price in November 2016).

Milk fat depression theories

Early theories

Many theories have been proposed to explain diet-induced MFD, including the acetate deficiency theory and glucogenic-insulin theory. Acetate is an energy and carbon source for milk fat synthesis in the mammary gland. In the acetate deficiency theory, diet-induced MFD is caused by a reduction in acetate production when cows are fed high concentrate and low forage diets (Balch et al., 1959). However, some studies only reported changes in rumen VFA molar proportions by treatments, which are not necessarily the same as alterations in actual production (Bauman and Griinari, 2001). Additionally, diet-induced MFD results in reduction of yields of

both de novo-synthesized FA and preformed FA (Baumgard et al., 2001; Rico and Harvatine, 2013). Therefore, the acetate deficiency theory does not fully explain diet-induced MFD. The glucogenic-insulin theory of MFD proposed that increased insulin inhibits body adipose tissue mobilization and leads to more energy and nutrients retained in adipose tissue, which results in a shortage of lipogenic precusors and energy available for milk fat synthesis (McClymont and Vallance, 1962; Jenny et al., 1974). Many studies have tested this theory by infusing glucose or propionate or administering a hyperinsulinemic-euglycemic clamp. However, those treatments only resulted in minor reductions in milk fat yield compared with diet-induced MFD. Those studies also demonstrated decreases in milk long chain FA and increases in de novo synthesized FA, which is contradicting to what is seen in diet-induced MFD (Bauman and Griinari, 2001).

Biohydrogenation theory

Bauman and Griinari (2001) were the first to propose the BH theory of MFD. They suggested that specific FA intermediates produced by altered BH pathways escape from the rumen and inhibit milk fat synthesis in mammary gland. Early studies tested the effects of mixed conjugated FA on milk fat synthesis and established a relationship between BH intermediates and MFD (Loor et al., 1998; Chouinard et al., 1999ab). Infusions of pure conjugated FA revealed that several BH intermediates can reduce milk fat synthesis and cause MFD, including *t*10, *c*12 CLA; *c*10, *t*12 CLA; and *t*9, *c*11 CLA (Baumgard et al., 2001; Sæbø et al., 2005; Perfield et al., 2007). Among these intermediates, *t*10, *c*12 CLA is the most well known and studied, with the other two only being tested in a single study and at a single dose.

Previous studies have reported a curvilinear relationship between abomasal infusion of *t*10, *c*12 CLA and percentage reduction in milk fat yield (Shingfield and Griinari, 2007). Milk fat synthesis involves the coordination of many lipid synthesis-related enzymes in the mammary

gland and yields of both de novo-synthesized and preformed milk FA are decreased during MFD (Baumgard et al., 2001; Rico and Harvatine, 2013; Boerman and Lock, 2014). *T*10, *c*12 CLA reduces lipid synthesis by inhibiting gene expression of several lipogenic enzymes including FA synthase, acetyl-CoA carboxylase, lipoprotein lipase, Δ9-desaturase, fatty acyl-CoA ligase, glycerol-phosphate-acyl-transferase, and acyl-glycerol-phosphate-acyl-transferase, and decreasing their mRNA abundance (Bauman et al., 2011). Reduced transcriptional activation of lipogenic genes is a result of *t*10, *c*12 CLA inhibiting proteolytic activation of sterol response element-binding protein-1 (SREBP-1; Peterson et al., 2004), which is considered a global lipid regulator (Shimano, 2009). Bauman et al. (2011) summarized the biological responses to *t*10, *c*12 CLA in the dairy cow. *T*10, *c*12 CLA has minor effects on feed intake, and no effects on milk protein, lactose, glucose set-point, basal insulin, ketogenesis, or liver lipids. *T*10, *c*12 CLA has been shown to up-regulate the expression of enzymes and key regulators of lipid synthesis in adipose tissue of dairy cows (Harvatine et al., 2009).

Two conditions are required for diet-induced MFD to occur: 1) altered rumen environment and rumen microbial population, and 2) dietary source of polyunsaturated FA (PUFA) (Bauman et al., 2011). Diet-induced MFD is often seen when cows are fed diets containing a large amount of highly digestible carbohydrate and a small amount of forage or when diets are supplemented with highly unsaturated oil. These diets change the rumen environment and rumen microbial population, and shift BH from the pathway producing *t*11 FA to the pathway producing *t*10 FA (Weimer et al., 2010; Zened et al., 2013; Rico et al., 2015). In bacteria culture studies, *Megasphaera elsdenii* and *Propionibacterium acnes* are able to isomerize LA to produce *t*10, *c*12 CLA (Kim et al., 2002; Wallace et al., 2007). In particular, *Megasphaera elsdenii* has been shown to be more abundant in cows exhibiting MFD than in

non-MFD cows (Palmonari et al., 2010; Weimer et al., 2010). Weimer et al. (2015) attempted to establish a robust population of *Megasphaera elsdenii* by dosing in order to induce MFD, but the dosed strain did not establish successfully.

Intermediates of BH are absorbed in the intestine, and transported to the mammary gland where they are incorporated into milk fat. *Trans* FA are markedly increased in milk fat during both *t*10, *c*12 CLA-induced and diet-induced MFD (Baumgard et al., 2001; Peterson et al., 20003; Boerman and Lock, 2014). A meta-analysis showed that extent of MFD is positively correlated ($R^2 = 0.63$) with percentage of *t*10 18:1 in milk fat in 31 studies with treatments consisting of high concentrate diets with or without unsaturated oils, or mixed diets supplementing with fish oil (Loor et al., 2005). It has been proposed that *t*10 18:1 could be one of the inhibitors of milk fat synthesis produced during rumen BH. Although the concentration of *t*10 18:1 increased markedly in milk fat, abomasal infusion of *t*10 18:1 at a dose of 42.6 g/d did not result in reduced milk fat yield or content (Lock et al., 2007). This indicates that production of *t*10 18:1 in rumen within the typical physiological range does not cause inhibition of milk fat synthesis during diet-induce MFD (Lock et al., 2007). Although *t*10 18:1 is not an inhibitor of milk fat synthesis, it is possible to use the concentration of *t*10 18:1 in milk as a marker for rumen BH shifts and MFD.

Factors altering biohydrogenation and MFD

Rumen pH

Low rumen pH has a negative impact on nutrient digestion, health and performance of dairy cows. Allen (1997) summarized the relationship between milk fat concentration and mean rumen pH across 23 published studies. Milk fat concentration was highly correlated with mean rumen pH ($R^2 = 0.39$, $P < 0.0001$), which indicates that reduced milk fat concentration is

associated with decreased mean rumen pH. Previous studies have used in vitro methods to test the effect of pH (range 5.5-6.78) on the BH of UFA (AbuGhazaleh et al., 2005; Fuentes et al., 2011; Troegeler-Meynadier et al., 2013). They reported that low rumen pH inhibits the BH of LA, decreases the formation of *c*9, *t*11 CLA, and increases the formation of *t*10, *c*12 CLA after short-term (< 8 h) incubations (Fuentes et al., 2011; Troegeler-Meynadier et al., 2013).

Low rumen pH inhibits the growth of rumen microbes, and thus, alters the rumen microbial population (Russell and Dombrowski, 1980; Russell and Wilson, 1996). This, subsequently, causes a shift in BH pathways (Fuentes et al., 2009). Rumen microbes are sensitive to pH changes, and different microbial species exhibit different levels of sensitivity to pH. Pure culture studies show that cellulolytic bacteria, such as *Ruminococcus albus*, *Bacteroides succinogenes* and *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens*, stop growing when pH drops below 5.7 (Russell and Dombrowski, 1980). However, lactic acid-utilizing bacteria, such as *Megasphaera elsdenii*, are more tolerant of low pH and can grow until culture pH drops to 4.9 (Russell and Dombrowski, 1980). *Butyrivibrio fibrisolvens* isomerizes and hydrolyzes LA, producing *c*9, *t*11 CLA and *t*11 18:1 as intermediates (Polan et al., 1964; McKain et al., 2010), and *Megasphaera elsdenii* isomerizes LA to form *t*10, *c*12 CLA (Kim et al., 2002). Compared with *Butyrivibrio fibrisolvens*, *Megasphaera elsdenii* is more competitive at low rumen pH, which may result in the shift of BH pathway. Low pH also inhibits the second reduction of *trans* 18:1 that forms saturated 18:0 (Trogegeler-Meynadier et al. 2006). *Butyrivibrio proteoclasticus* hydrogenates *trans* 18:1 to 18:0 (Wallace et al., 2006) and may also be sensitive to low pH. A continuous culture study reported that, compared with pH 6.4, pH 5.6 decreased DNA concentrations of both *Anaerovibrio lipolytica* and *Butyrivibrio* spp., flow of 18:0, *t*11 18:1, and *c*9, *t*11 CLA, and increased flow of *t*10 18:1, *t*10, *c*12 CLA and LA (Fuentes et al., 2009).

Although rumen microbes are sensitive to pH and changes in rumen pH can have a great impact on the microbial population, cows exhibiting different pH dynamics can still have similar rumen microbial populations (Palmonari et al., 2010). Additionally, among a group of cows, MFD was observed in the ones with relative intermediate rumen pH (6.30), but not in the high (6.51) or low (6.11) rumen pH cows (Palmonari et al., 2010). This result indicates that cows with intermediate rumen pH may also exhibit MFD.

Dietary UFA

Dietary unsaturated FA are required for diet-induced MFD to occur (Bauman et al., 2011). Supplementation of UFA-enriched vegetable oil is commonly used in studies to induce MFD (Rico and Harvatine, 2013; Rico et al., 2014bc). LA was shown to be a more potent inhibitor of milk fat synthesis than OA when total FA was consistent across two diets (He et al., 2012). Additionally, even though total dietary FA was less than 3%, supplementing oil rich in LA still reduced milk fat yield primarily by inhibiting de novo FA synthesis (Stoffel et al., 2015).

An in vitro batch culture study tested the effect of increasing LA (additions of 1.0 to 10.0 mg per culture) on disappearance and formation of BH intermediates (Honkanen et al., 2012). Over 90% of added LA was hydrogenated, and increasing LA resulted in the accumulation of both *c*9, *t*11 CLA and *t*10, *c*12 CLA. Notably, *t*10, *c*12 CLA increased at a greater rate than did *c*9, *t*11 CLA (Honkanen et al., 2012). The addition of LA may inhibit the first isomerisation of LA, and the subsequent accumulation of BH intermediates may inhibit or saturate the first and second hydrogenation steps that form *trans* 18:1 and 18:0 (Troegeler-Meynadier et al., 2006). Rumen bacteria may be able to increase BH capacity in order to compensate for increasing UFA load. Increasing the amount of LA from 100 to 300 mg in an in vitro culture system increased the disappearance of LA without changing the ratio of *t*10:*t*11 (Troegeler-Meynadier et al., 2003).

UFA are toxic to rumen bacteria and inhibit their growth (Maia et al., 2010). BH is a process that rumen bacteria convert dietary UFA to saturated FA. The toxicity of UFA toward rumen bacteria increases with increasing numbers of double bonds (eicosapentaenoic acid [20:5] > docosahexaenoic acid [22:6] > LNA [18:3] > LA [18:2]) (Maia at el., 2007). The toxicity of UFA for rumen bacteria may be the result of disruption of UFA double bonds on the lipid bilayer structure of bacterial membranes (Keweloh and Heipeiper, 1996). Additionally, UFA may inhibit the growth of rumen bacteria by disrupting bacterial metabolism (Maia et al., 2007 and 2010). Bacteria producing butyrate through butyrate kinase appear to be more sensitive to UFA than others (Maia et al., 2010). Therefore, rumen bacteria of different species exhibit different sensitivities to UFA. Both cellulolytic bacteria, including *Butyrivibrio fibrisolvens*, and other butyrate-producing bacteria, such as *Butyrivibrio proteoclasticus*, *Butyrivibrio hungatei*, did not grow in culture supplemented with PUFA at 50 μg/mL (Maia et al., 2007). On the other hand, some bacteria, including *Megasphaera elsdenii* and *Anaerovibrio lipolytica*, are insensitive to UFA (Maia et al., 2007). The toxicity of UFA toward cellulolytic bacteria is considered one explanation for the fact that oil supplements reduce NDF digestibility, in addition to the coating effect of oil on fiber that prevents adhesion of rumen microbes (Palmquist and Jenkins, 1980).

Dietary fermentability

To increase energy intake, dairy cows are often fed highly fermentable diets containing low fiber and high starch content or highly rumen degradable starch. Diets containing large amounts of highly fermentable carbohydrate usually cause MFD (Firkins et al., 2001; Bauman et al., 2011), and have been used experimentally to induce MFD (Longuski et al., 2009). A meta-analysis reported that increasing dietary starch content or starch digestibility was associated with reduced milk fat content (Ferraretto et al., 2013). Additionally, dietary starch content and starch

fermentability interact to influence milk fat synthesis. Oba and Allen (2003) reported that feeding cows high moisture corn in a high-starch diet led to decreased milk fat concentration compared with dry ground corn; however, these starch sources did not affect milk fat concentration differently in low-starch diets.

Increasing starch degradability and content inhibit BH of UFA and cause shifts in BH pathways (Gerson et al., 1985; Zened et al. 2012 and 2013). Increasing starch degradability while holding starch level constant resulted in decreased extent of BH of LA and formation of *t*11 18:1, and *c*9, *t*11 CLA, and increased *t*10 18:1 and *t*10, *c*12 CLA (Lascano et al., 2016). A short-term (≤ 2 h) in vitro culture study tested the effect of carbohydrate fermentability and content on TAG hydrolysis and UFA hydrogenation by using rumen digesta from sheep fed different diets, and found that increasing dietary starch content decreased both hydrolysis and BH rate (Gerson et al., 1985). Starch content affects growth of rumen bacteria (Cotta, 1988; Fuentes et al., 2009). Cotta (1988) reported that a starch-containing medium had different effects on growth rate of selected rumen bacteria species including *Bacteroides ruminicola*, *Streptococcus bovis*, and *Butyrivibrio fibrisolvens*. Additionally, dietary concentrate level has been shown to interact with pH to influence DNA concentrations of bacteria involved in lipolysis and BH (Fuentes et al., 2009). High pH increased *Anaerovibrio lipolytica* compared with low pH, and the increase was greater in high concentrate diet than low concentrate diet; low pH generally decreased *Butyrivibrio* spp. compared with high pH, and the reduction was greater in high concentrate diet than low concentrate diet (Fuentes et al., 2009).

Highly fermentable diets occasionally cause reductions in rumen pH (Sutton, 1977; Bauman and Griinari, 2003) because of increased production of acids in the rumen and decreased VFA absorption rates and secretion of salivary buffer (Allen, 1997). Low rumen pH changes the

rumen microbial population, causes shifts in rumen BH, and therefore, may result in MFD (Palmonari et al., 2010; Fuentes et al., 2011; Troegeler-Meynadier et al., 2013). Therefore, diets containing high starch content and highly fermentable starch may cause MFD in dairy cows by reducing rumen pH (Van Nevel and Demeyer, 1996; Zened et al., 2013). It is not clear whether dietary starch is the main factor that shifts rumen BH from forming *t*11 FA to forming *t*10 FA, or if the shift is the result of reduced rumen pH. One study found that compared with a low-concentrate treatment (forage:concentrate = 70:30), a high-concentrate treatment (forage:concentrate = 30:70) increased concentration of *t*10, *c*12 CLA in culture 1 h after feeding, and the increased concentration of *t*10, *c*12 CLA was less than the one associated with low pH (pH 5.6; Fuentes et al., 2009). Therefore, decreased rumen pH may be the primary cause of *t*10, *c*12 CLA formation, with increased concentrate playing a minor role (Fuentes et al., 2009). However, another in vitro study reported that, compared with low dietary starch content (100% lucerne hay), high starch content (50% lucerne hay and 50% starch) increased the concentration of *t*10 18:1 in culture regardless of pH (6.0 or 7.0), but did not influence *t*10, *c*12 CLA. The authors concluded that the presence of increased starch, rather than decreased rumen pH, caused the shift in BH (Maia et al., 2009).

Saccharomyces cerevisiae fermentation product

A recent meta-analysis showed that a *Saccharomyces cerevisiae* fermentation product (SCFP) increased milk fat yield (Poppy et al., 2012). Longuski et al. (2009) reported that SCFP prevented MFD when diet fermentability was altered over a short period of time. Several potential mechanisms may explain the mechanism by which SCFP prevents MFD, including the effect of the supplement on the metabolism of rumen microbes and stabilization of fermentation (Harrison et al., 1988; Miller-Webster et al., 2002).

SCFP enhanced total VFA production and shifted the molar proportions of VFA toward propionic acid in a continuous culture system (Miller-Webster et al., 2002). Previous studies reported that SCFP changed the rumen bacteria population and stimulated growth of some bacteria, especially cellulolytic bacteria, in vitro (Callaway and Martin, 1997; Harrison et al., 1988; Newbold et al., 1995). Cows supplemented with SCFP exhibited more stable rumen fermentation and higher rumen cellulolytic bacteria concentration (Harrison et al., 1988). A rumen bacteria culture study showed SCFP stimulated the growth of *Fibrobacter succinogenes* and *Ruminococcus albus* in a medium containing 6 g/L of cellobiose (Callaway and Martin, 1997). However, one study, which analyzed rumen content collected from SCFP-supplemented cows, found no effects of supplementation on tested microbial species including *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Ruminococcus albus*, and *Megasphaera elsdenii* etc (Mullin et al., 2013). Diet ingredients affected rumen microbial populations and fermentation (Boguhn et al., 2012), and the effect of these ingredients may have mitigated the effects of SCFP on the rumen microbiome in Mullin et al. (2013). It indicates that SCFP may have interacted with dietary factors to affect rumen bacterial populations, thus, subsequently influence BH pathway. However, limited information is available on the specific effects of SCFP on BH pathways and formation of BH intermediates associated with MFD.

Production level of dairy cows

Interactions between production level of dairy cows and diets, differing in starch concentration or fermentability, have been observed in previous studies (Voelker et al., 2002; Bradford and Allen, 2003; Boerman et al., 2015). Cows at different production levels may respond differently to diets that induced MFD via different mechanisms. However, there is limited research in this field. Moreover, the available results have been inconsistent and the

mechanisms were not resolved. When cows were fed high-starch diets (> 32% DM) containing high moisture corn or dry ground corn, low producing cows exhibited decreased milk fat concentration, while high producing cows showed no change in milk fat (Bradford and Allen, 2003). However, a diet supplemented with 2.3% Ca-salts of palm FA reduced milk fat concentration in high producing cows, but not in low producing cows (Rico and Harvatine, 2014c). Therefore, differing treatment diets could result in opposing results via different mechanisms. High-starch diets often cause reductions in rumen pH (Allen, 1997). High producing cows are better able to absorb VFA and stabilize their rumen environment compared to low producing cows (Voelker et al., 2002). Therefore, high producing cows may have been able to maintain higher rumen pH, and thus mitigate the negative impacts on rumen BH when they were fed a highly fermentable diet (Bradford and Allen, 2003). On the other hand, high producing cows have higher rumen passage rates than lower producing cows, which may have resulted in more FA intermediates associated with MFD passing from the rumen and leading to MFD (Rico et al., 2014c). However, neither study tested possible mechanisms. Therefore, there is a need to test the interaction between production level and diet-induced MFD using treatment diets with common dietary risk factors for MFD, including high dietary starch content, starch fermentability, and UFA content.

Techniques utilized in BH and MFD studies

In vitro culture techniques

In vitro batch and continuous culture incubation techniques are widely used to study rumen fermentation and microbial metabolism because of the advantages of low cost and flexible, well-controlled conditions compared with in vivo techniques (Boguhn et al., 2014; Zened et al., 2011; Vlaeminck et al., 2008). A 125-mL Erlenmeyer flask vessel is typically used in in vitro

artificial rumen procedures (Goering and Van Soest, 1970). For practical reasons, vessels of different volumes and shapes have been tested in in vitro experiments (Sayre and Van Soest, 1972). In one study, large glass tubes (200 × 25 mm) yielded similar NDF digestibility results compared with the traditional 125-mL Erlenmeyer flask vessels (Sayre and Van Soest, 1972).

However, in vitro studies may not be comparable due to the variation in rumen inoculum, which affected by donor cow nutrition, inoculum collecting time and source, and preparation and inoculation of inoculum (Mould et al., 2005). Therefore, it is important to follow a relatively consistent and standard protocol for rumen inoculum collection and preparation (Mould et al., 2005). Additionally, due to the stability and specificity of the microbial community, the new rumen, microbial equilibrium would not be established until days after a dietary change (Weimer et al., 2010). Typical cultures are incubated for 24 h or less in batch culture studies (Van Nevel and Demeyer, 1996; Choi and Song, 2005; Troegeler-Meynadier et al., 2006). Therefore, it is not feasible to test effects on BH by adding supplements directly to batch cultures. Previous studies utilized rumen inoculum collected from donor cows adapted to treatment diets for 14 or 21 d to test the effects of dietary factors or supplements and rumen conditions on BH (Vlaeminck et al., 2008; Zened et al., 2011)

Treatment diets of in vivo studies

Several studies have successfully induced MFD with diets containing varied ingredients and nutrient compositions (Rico and Harvatine, 2013; Ramirez Ramirez et al., 2015; Ma et al., 2015). Diets with increased dietary starch or supplemental plant or fish oil are commonly used to induce MFD. Holding other feed ingredients constant, He and Armentano (2011) replaced corn starch in a control diet with 5% mixed vegetable oil to induce MFD. Rico and Harvatine (2013) and Ma et al. (2015) induced MFD with diets contained less forage than a control diet and

supplemented soybean oil or fish oil. Hötger et al. (2013) supplemented rumen-protected *t*10, *c*12 CLA to evaluate glucose metabolism in dairy cows during MFD. Glasser et al., (2010) fed dairy cows a high concentrate diet (forage:concentrate = 35:65) to compare the effects of *t*10, *c*12 CLA induced-MFD and a high concentrate-induced MFD on milk fat synthesis. Although these diets all induced MFD, the nutrient compositions and supplements used are not common on commercial dairy farms. Therefore, there is a need to study diet-induced MFD using practical treatment diets.

Conclusion

Although the effects of individual dietary factors and rumen pH on BH of UFA have been extensively described in previous studies, there are still substantial gaps in our knowledge concerning the interactions between these factors. Few studies have tested interactions between rumen pH and dietary factors including UFA, starch content, and starch fermentability on BH pathways. Furthermore, the on-farm relevance of these in vitro results may be limited by factors including the lack of nutritive substrate in cultures (Van Nevel and Demeyer, 1996), single-content starch in cultures (Lascano et al., 2016), and extreme variation in pH treatments (Fuentes et al., 2009). Therefore, improvements in study methods and treatments are necessary to elucidate a comprehensive understanding of the complex interactions among MFD risk factors. Our objectives included: 1) assessing the effects of UFA concentration, starch content and starch fermentability on BH pathways at two pH levels, 2) determining the effects of *Saccharomyces cerevisiae* fermentation product on BH pathways under varied culture pH, starch content, and starch fermentability conditions, and 3) assessing the variation in cow responses to diet-induced MFD across a wide range of production levels. Our central hypothesis is that MFD risk factors interact to influence rumen BH pathways and milk fat synthesis, and low rumen pH will emerge

as the predominant risk factor involved in MFD. Our rationale for these studies is that the results will allow us to develop effective dietary strategies for preventing MFD on dairy farms and maximizing milk fat yield and farm income.

APPENDIX

APPENDIX

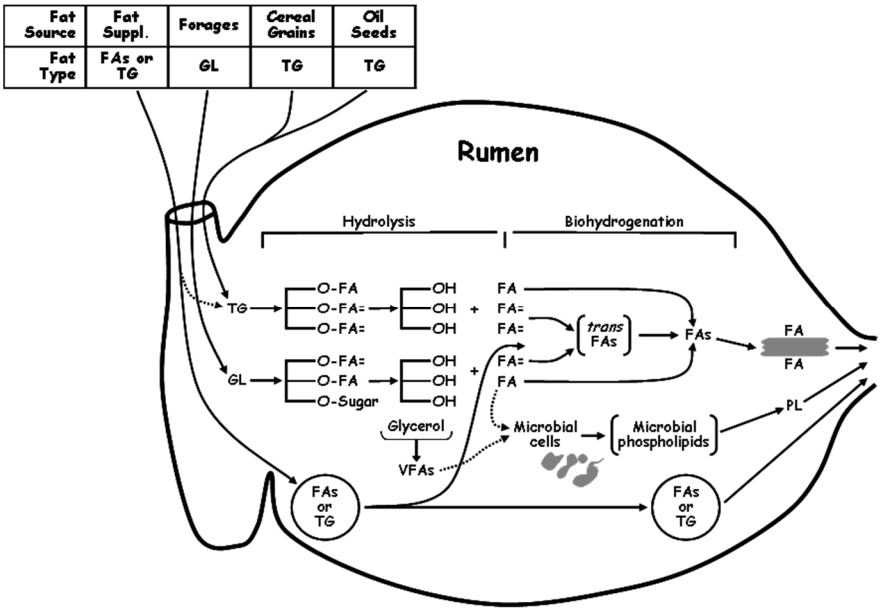

Figure 1.1. Lipid metabolism in the rumen.

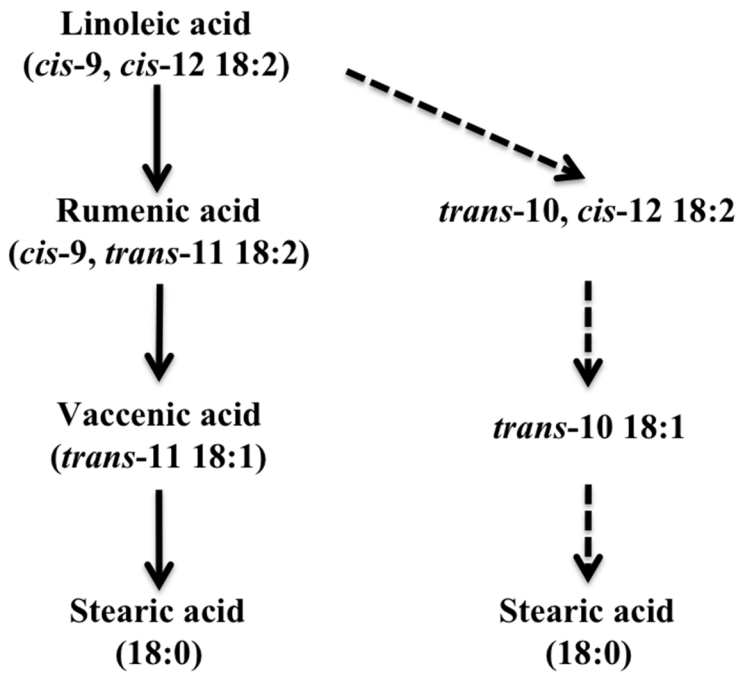

Figure 1.2. Biohydrogenation pathways of linoleic acid.
Pathways in figure include dominant pathway under normal conditions (left side) and alternative pathway during diet-induced MFD (dotted lines, right side).

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CHAPTER 2

INTERACTION BETWEEN CULTURE PH AND CORN OIL CONCENTRATION ON NEUTRAL DETERGENT FIBER DIGESTIBILITY AND BIOHYDROGENATION OF UNSATURATED FATTY ACIDS IN BATCH CULTURE

INTRODUCTION

Milk fat yield is a major determinant of milk income. Compared with other milk components, fat is the most sensitive to changes in diets and environment. Diet-induced milk fat depression (MFD) is defined as an up to 50% reduction in milk fat yield with minor changes in other milk components and total milk yield (Bauman et al., 2011). In past decades, researchers found that diet-induced MFD is mainly caused by the inhibition of milk fat synthesis in the mammary gland by specific rumen PUFA biohydrogenation (BH) intermediates (Bauman et al., 2011). Rumen bacteria biohydrogenate unsaturated FA in the rumen and produce various FA intermediates (Shingfield and Wallace, 2014). Among known BH intermediates, *t*10, *c*12 conjugated linoleic acid (CLA) has been well characterized as a potent inhibitor of milk fat synthesis (Baumgard et al., 2001), and *t*10 18:1 in milk is correlated with reduction in milk fat concentration (Loor et al., 2005; Kadegowada et al., 2008).

Linoleic acid (LA, *c*9, *c*12 18:2) is the most abundant dietary FA for dairy cows. Rumen bacteria biohydrogenate LA with *c*9, *t*11 CLA and *t*11 18:1 as major intermediates. However, other intermediates are also produced during BH of LA (Honkanen et al., 2012). Changes in diet and the rumen environment can cause more LA to be converted to *t*10, *c*12 CLA and *t*10 18:1 (Bauman et al., 2011). Rumen pH and milk fat percentage are highly positively correlated (Allen, 1997), potentially due to inhibition of rumen bacteria growth (Russell and Dombrowski, 1980). Several in vitro studies have tested the effects of pH on BH of unsaturated FA and shown that low pH inhibited BH and increased formation of *t*10, *c*12 CLA (Troegeler-Meynadier et al.,

2003; Fuentes et al., 2011; Troegeler-Meynadier et al., 2013). Previous in vitro incubation studies have found that increasing LA inhibits complete BH of LA to 18:0 (Troegeler-Meynadier et al., 2003; Honkanen et al., 2012), potentially through the inhibition of *Butyrivibro fibrisolvens* growth (Kim et al., 2000). However, most published studies do not take into account the interactions between dietary and ruminal factors. Typically, dietary changes will affect rumen environment, and the combinations of those alterations can increase the risk of MFD. Van Nevel and Demeyer (1996) determined the influence of pH and soybean oil level on triglyceride hydrolysis and BH in vitro, and found that hydrolysis was more sensitive to low pH than BH especially with the larger amount of soybean oil. However, no nutritive substrate other than soybean oil was included in the cultures, and no specific FA intermediates of BH were reported. The presence of a fibrous substrate is essential to accurately represent the complex metabolism in rumen, and quantification of BH intermediates is necessary to discern the possible effects of these rumen factors on milk fat synthesis. Therefore, additional experimentation is necessary to predict the impact of rumen pH and dietary unsaturated FA on the production of BH intermediates associated with MFD. The objective of our study was to test the interactions of pH and corn oil, at common dietary concentrations, on BH by using an in vitro batch culture method. We hypothesized that there would be interactions between rumen pH and corn oil concentration on BH of LA. Furthermore, we hypothesized that increasing LA at a low pH would cause greater inhibition of BH, compared to a high pH environment, and increase formation of *t*10, *c*12 CLA and *t*10 18:1.

MATERIAL AND METHODS

Treatment and Incubation

In vitro batch cultures were set up in a 2 × 3 factorial arrangement of treatments: two culture pH levels (low pH = 5.8 or high pH = 6.2), and three corn oil (OIL) concentrations (0, 1, or 2%). All cultures were run in quadruplicate, with two analyzed for neutral detergent fiber (NDF) residue and two for FA composition. All cultures contained alfalfa hay as the base substrate, which was dried at 55ºC for 48 h and ground through a 1-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA). The 0% corn oil treatment was alfalfa hay containing no corn oil. Rather than adding oil in the culture, alfalfa hay containing 2% corn oil on a DM basis was prepared by spraying corn oil in ethanol onto the corresponding weight of alfalfa hay. The alfalfa hay plus corn oil was mixed well during spraying, dried at 55ºC, and ground through a 1-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA) for consistency. Alfalfa hay containing 1% corn oil was prepared by combining alfalfa hay with prepared alfalfa hay containing 2% corn oil on 1:1 ratio. FA content and profile were analyzed for alfalfa hay containing OIL (0, 1, or 2%) after grinding, and results are shown in Table 2.1.

Three rumen-fistulated mid-lactation Holstein dairy cows (185 ± 9 DIM) fed a common TMR (27% corn silage, 14% haylage, 20% ground corn, 17% soybean meal, 7% high moisture corn, 2% soy hulls, 3% wheat straw, 7% cottonseed, 3% mineral-vitamin) were used as rumen inoculum donors for this study. Rumen fluid and solid digesta were collected 1 h after feeding and blended in a 1-gal Waring blender at low speed for 25 s to detach the bacteria from the feed particles. The blended mixture was then passed through a Buchner funnel lined with nylon mesh and glass wool to trap large particles, which were discarded. The strained rumen fluid was

transferred to a 500-mL Brinkman pipette bottle for inoculating individual culture tubes, and was continuously flushed with CO_2.

All cultures were prepared as described by Goering and Van Soest (1970). Sayre and Van Soest (1972) previously reported that using large glass tubes (200 × 25 mm) as incubation vessels yielded similar NDF digestibility results compared to 125-mL Erlenmeyer flask vessels, which are more typical for in vitro artificial rumen procedures. In the present study, Pyrex centrifuge tubes (100-mL) were used as incubation vessels. To prevent accumulation of acid produced by ruminal microorganisms, media solution was used to buffer pH during incubation. Volumes of 1 M citric acid were added into the media solution to obtain culture pH levels of 5.8 and 6.2, as described by Grant and Mertens (1992). Each vessel contained 500 mg of alfalfa hay containing 0, 1, or 2% corn oil, 40 mL of media solution (pH 5.8 or 6.2), 2 mL of reducing solution, and 10 mL of rumen fluid. Substrate weight of each culture was recorded for NDF disappearance extent calculation. Culture tubes were flushed with CO_2 and sealed with 5.5-cm rubber stoppers, connected to a Bunsen valve in order to release excessive gas during incubation. All cultures were incubated in the same water bath at 39ºC.

Incubation of sampled cultures was stopped at 0, 6, 12, 18, and 24 h of incubation. Immediately after rumen fluid inoculation, culture tubes taken at 0 h were placed in an ice bath and dry ice was added to stop biohydrogenation. At 6, 12, 18, and 24 h, incubations were also terminated as described above. The pH of all culture tubes was tested immediately before incubations were terminated. Culture tubes for NDF residue analysis (2 replicates) were placed in a 4ºC cooler until analysis (completed within 48 h). Culture tubes for FA composition analysis (2 replicates) were stored at -20ºC and subsequently freeze-dried.

Chemical Analyses

NDF residue was analyzed as described by Mertens (2002) to determine NDF disappearance extent. To ensure an accurate analysis of FA composition, samples were directly freeze-dried and methylated in culture tubes using a 2-step methylation protocol adapted from Jenkins (2010). Internal standard (17:0, 1:1 mg/mL toluene) was added to cultures after incubation was terminated and prior to storage at -20°C. To determine the dry culture content weight, all tubes were pre-weighed and re-weighed after being freeze-dried. Freeze-dried samples were mixed with 8 mL of 0.5 M sodium methoxide solution in methanol and incubated for 10 min in a 50°C water bath. After tubes cooled, 12 mL of 5% methanolic hydrochloric acid solution was added, followed by 10 min of incubation in a 80°C water bath. After tubes were removed from the water bath and cooled, 10 mL n-hexane and 30 mL 6% K_2CO_3 solution were added and the solution was mixed by vortexing. Tubes were centrifuged for 10 min at 3000 rpm and the hexane layer containing FAME was transferred to a 15-mL centrifuge tube containing 2 g sodium sulfate. FAME were extracted again by adding 5 mL hexane to the tubes and repeating the mixing, centrifuging, and transferring steps described above. The 15-mL centrifuge tubes were inverted and the samples were allowed to settle for 10 min. The solution was filtered through silica gel and charcoal to remove the sodium sulfate and the solvent was evaporated with a nitrogen gas at 37°C. The FAME samples were weighed and a 1% solution was prepared with n-hexane based on weight. The 1% FAME solution was transferred to 2-mL GLC vials for analysis.

FA composition was determined by a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with a split injector (1:100 split ratio) and a flame-ionization detector (FID) using a CP-Sill 88 WCOT (wall-coated open tubular) fused-silica column (100 m × 0.25 mm i.d. × 0.2-

μm film thickness; Varian Inc., Lake Forest, CA). The carrier gas was hydrogen at a flow rate of 1 mL/min. Hydrogen, purified air, and nitrogen makeup gas were used as the FID gases at flow rates of 40, 400, and 30 mL/min, respectively. Injector and detector temperature was 270ºC. The oven program was as described below: initial temperature held for 0.5 min at 40ºC, programmed to increase the temperature to 155ºC at 25ºC/min and held for 30 min, and then increased to 215ºC at 4ºC/min and held for 35 min. Injection volume was 1 μL. FID response was the basis for integration and quantification (GCsolution software version 2.32.00; Shimadzu). Known FAME standards (GLC reference standard 463, GLC reference standard 481-B, and conjugated octadecadienoic mixture #UC-59-M from Nu-Chek Prep Inc., Elysian, MN; Supelco 37 component FAME mix, cis/trans FAME mix, bacterial acid methyl ester mix, and PUFA No. 2 mix from Supelco Inc., Bellefonte, PA) were used for determination of individual FAME by comparing retention times. Quantification of FA composition covered approximately 45 FA in the range of 12:0 to 24:0.

Calculations and Statistical Analysis

NDF disappearance extent was calculated by subtracting the content of NDF in the cultures at 6, 12, 18, and 24 h from the content at 0 h and dividing by the content at 0 h. This provided the percentage of NDF disappearance at each time point compared to 0 h. The extent of biohydrogenation was calculated by subtracting the amount of LA in the cultures at 6, 12, 18, and 24 h from the amount at 0 h and dividing by the amount at 0 h. Similarly, this provided the percentage of LA disappearance at each time point compared to 0 h.

All data were analyzed using the fit model procedure of JMP (Version 10, SAS Institute). The pH, NDF disappearance extent, extent of BH, total FA content, and FA concentrations of in vitro batch cultures at 24 h of incubation were analyzed using a model that contained the main

effects of culture pH, OIL concentration, and the interaction between culture pH and OIL concentration. In order to test the treatment effect across time on FA composition, results obtained at each sampling time were analyzed separately using the same model but with the effect of time and interactions as main effects. Least square means with standard error are reported, and significance declared at $P < 0.05$.

RESULTS

FA profile and total FA content of substrates

Total FA content and FA profile of alfalfa with different OIL concentration are shown in Table 2.1. As expected increasing OIL increased total FA content and concentrations of *c*9 18:1 and LA, and decreased concentrations of 16:0, 18:0, and *c*9, *c*12, *c*15 18:3.

pH, NDF disappearance extent, and BH extent of in vitro cultures

Effects of treatment on pH, NDF disappearance extent, BH extent of LA, and total FA content after 24 h of incubation are shown in Table 2.2. Increasing OIL increased total FA content of cultures at both low and high pH ($P < 0.001$). Changes in these variables during the 24-h incubation are shown in Figures 2.1-2.3. Overall, the pH of all cultures fluctuated during incubation, and average pH difference between low pH and high pH cultures remained over 0.2 units across time (5.94 vs. 6.19). After 24 h of incubation, the average pH of low pH and high pH cultures were 6.06 and 6.21 ($P < 0.001$) respectively. Adding OIL had no effect on culture pH after 24 h of incubation. NDF disappearance extent increased during incubation, and was higher in high pH than low pH cultures across time (Figure 2.2). After 24 h of incubation, low pH decreased NDF disappearance extent compared with high pH (22.7 vs. 34.9%, $P < 0.001$). Increasing OIL increased NDF disappearance extent (25.4, 29.0, and 32.1%, respectively; $P < 0.001$). Similar to NDF disappearance extent, BH extent increased during incubation, and was

higher in low pH cultures than in high pH cultures (Figure 2.3). In high pH cultures, 0% OIL had lower BH extent than 1 and 2% OIL. After 24 h of incubation, low pH decreased BH extent compared with high pH (52.2 vs. 76.1%, $P < 0.001$). Adding OIL increased BH extent, and there was no difference between 1% OIL and 2% OIL. We observed interactions between pH and OIL ($P < 0.001$). At low pH, 1% OIL increased BH extent compared with 0 and 2% OIL ($P < 0.001$); at high pH, adding OIL increased BH extent compared with 0% OIL ($P < 0.001$), and there was no difference between 1% and 2% OIL.

Effects of culture pH and OIL on FA profile of cultures after 24 h of incubation

Results follow a similar pattern for FA if reported as g/100 g FA or mg/culture. Therefore, we only report the results for FA profile and focus on individual FA associated with BH of LA. Table 2.3 shows the effects of pH and OIL on FA concentrations in cultures after 24 h of incubation. Overall, compared with high pH, low pH decreased the concentrations of total SFA (47.4 vs. 57.9 g/100 g FA, $P < 0.001$), total *trans* 18:1 FA (12.1 vs. 13.2 g/100 g FA, $P < 0.001$), and total odd- and branched-chain FA (OBCFA; 4.87 vs. 5.83 g/100 g FA, $P < 0.001$), while increasing concentrations of MUFA (14.5 vs. 10.8 g/100 g FA, $P < 0.001$) and PUFA (14.7 vs. 6.78 g/100 g FA, $P < 0.001$). Compared with high pH, low pH increased concentrations of LA (12.1 vs. 5.05 g/100 g FA, $P < 0.001$), *c*9, *t*11 CLA (0.25 vs. 0.20 g/100 g FA, $P < 0.001$), *t*10, *c*12 CLA (0.14 vs. 0.07 g/100 g FA, $P < 0.001$), and *t*10 18:1 (1.71 vs. 0.95 g/100 g FA, $P < 0.001$), and decreased concentrations of *t*11 18:1 (8.62 vs. 10.3 g/100 g FA, $P = 0.001$) and 18:0 (21.2 vs. 31.0 g/100 g FA, $P < 0.001$).

Overall, increasing OIL increased concentrations of total *trans* 18:1 FA (6.85, 13.8, and 17.4 g/100 g FA; $P < 0.001$), MUFA (8.42, 13.3, and 16.2 g/100 g FA, $P < 0.001$), and PUFA (7.85, 10.7, and 13.7 g/100 g FA; $P < 0.001$), and decreased concentrations of SFA (62.4, 51. 5,

and 44.0 g/100 g FA; $P < 0.001$) and OBCFA (7.44, 4.97, and 3.64 g/100 g FA; $P < 0.001$) for 0, 1, 2% respectively. Increasing OIL increased concentrations of LA (4.84, 8.74, and 12.1 g/100 g FA, $P < 0.001$), *c*9, *t*11 CLA (0.06, 0.26, and 0.36 g/100 g FA, $P < 0.001$), *t*10, *c*12 CLA (0.03, 0.10, and 0.17 g/100 g FA, $P < 0.001$), *t*11 18:1 (4.73, 10.4, and 13.2 g/100 g FA, $P < 0.001$), *t*10 18:1 (0.78, 1.41, and 1.80 g/100 g FA, $P < 0.001$), and decreased 18:0 (30.2, 26.2, and 22.0 g/100 g FA, $P < 0.001$) for 0, 1, 2% respectively.

Figure 2.4 shows the interaction between culture pH and OIL on concentrations of *t*10, *c*12 CLA and *t*10 18:1. Increasing OIL linearly increased concentrations of *t*10, *c*12 CLA and *t*10 18:1 at both low and high pH. However, rate of change of *t*10, *c*12 CLA and *t*10 18:1 are greater at low pH (0.10 and 0.64, respectively) than high pH (0.04 and 0.38, respectively) with increasing OIL. Compared with 0%, OIL at 1% and 2% of DM increased LA by 120 and 225% at low pH ($P < 0.001$), respectively. At high pH, 2% OIL increased LA by 46% ($P < 0.001$), but there was no difference between 0 and 1% OIL. Compared with 0% OIL, 1 and 2% of OIL decreased 18:0 by 28 and 45% at low pH ($P < 0.001$), respectively. At high pH, 2% OIL decreased 18:0 by 11% ($P < 0.001$), but there was no significant difference between 0 and 1% OIL. Compared with 0%, 1 and 2% OIL increased *t*11 18:1 by 93 and 108% at low pH ($P < 0.001$), respectively, and by 151 and 266% at high pH ($P < 0.001$), respectively.

Changes in FA associated with BH of LA in cultures during 24 h of incubation

The effects of culture pH and OIL over time on FA associated with BH of LA are shown in Figures 2.5 to 2.7. LA decreased during incubation in both low and high pH cultures, and decreased faster at high pH than low pH. However, 18:0 increased during incubation in high pH cultures ($P < 0.001$), and it had minor changes in low pH cultures only after 18 and 24 h of incubation ($P = 0.003$). *C*9, *t*11 CLA increased during the first 12 h of incubation and decreased

during the second 12 h of incubation in cultures with OIL at high pH (Figure 2.6). *C*9, *t*11 CLA increased during 24 h of incubation in cultures with OIL at low pH. Cultures not containing OIL exhibited decreased *c*9, *t*11 CLA during incubation at both low and high pH. Generally, *t*11 18:1 increased in all cultures, but this FA increased faster in cultures containing OIL. Furthermore, *t*11 18:1 increased faster in cultures at high pH than those at low pH.

*T*10, *c*12 CLA increased in cultures with OIL at low pH during 24 h of incubation (Figure 2.7). Furthermore, *t*10, *c*12 CLA increased during the first 18 h of incubation and decreased during the last 6 h of incubation in cultures with OIL at high pH. Cultures without OIL exhibited decreased *t*10, *c*12 CLA after 18 h of incubation for cultures at low pH and after 6 h of incubation for cultures at high pH. Following 24 h of incubation, cultures containing 2% OIL at high pH had similar *t*10, *c*12 CLA to those containing 1% OIL at low pH. Generally, *t*10 18:1 increased in all cultures during incubation, except those containing no OIL at high pH, which actually decreased in *t*10 18:1 concentration. Cultures at low pH, regardless of OIL concentration, increased *t*10 18:1 content faster than cultures at high pH. Cultures containing 2% OIL at high pH ended the incubation period with lower *t*10 18:1 concentrations than cultures containing 1% OIL at low pH.

DISCUSSION

Dietary PUFA are toxic to rumen bacteria, possibly by influencing membrane integrity or inhibiting bacterial metabolism (Maia et al., 2007). Rumen bacteria biohydrogenate PUFA to SFA through several isomerization and hydrogenation steps, and this process reduced toxicity of UFA. Numerous FA intermediates are formed in these processes (Griinari and Bauman, 1999). Linoleic acid (*c*9, *c*12 18:2) is the most abundant PUFA in most cow rations based on corn and soy. Among all intermediates, *c*9, *t*11 CLA and *t*11 18:1 are the major intermediates produced

during BH of LA (Griinari and Bauman, 1999). However, specific dietary and rumen environment changes often cause more LA to be biohydrogenated through the pathway producing *t*10, *c*12 CLA and *t*10 18:1 as intermediates. It has been well documented that *t*10, *c*12 CLA exhibits bioactive functions, including the inhibition of milk fat synthesis (Baumgard et al., 2001). Similarly, *t*10 18:1 is negatively correlated with milk fat content and yield, and is commonly used as a marker for MFD (Loor et al., 2005; Kadegowada et al., 2008). Diet-induced MFD is often caused by the interaction of several risk factors, rather than a single diet characteristic. Diet-induced MFD requires the presence of both dietary PUFA and changes in rumen microbial populations or rumen environment, including alteration of pH (Bauman and Griinari, 2003). Our study utilized OIL as a PUFA source and investigated the effect of the interaction between culture pH and OIL concentration on NDF disappearance extent and biohydrogenation of LA.

Several in vitro studies have tested the effects of pH on BH of PUFA across a wide range (pH=5.5-6.78) (Fuentes et al., 2011; AbuGhazaleh et al., 2005; Troegeler-Meynadier et al., 2013). However, the range of tested pH levels was too wide and therefore not suitable for evaluating the sensitivity of BH to normal rumen pH variation. Low pH inhibits the growth of rumen bacteria and rumen nutrient digestibility (Russell and Dombrowski, 1980; Russell and Wilson, 1996), and pH 5.8 is often chosen as a threshold for subacute acidosis (Beauchemin and Yang, 2005; Dohme et al., 2008; Mohammed et al., 2012). Therefore, we utilized a small pH range, 5.8 to 6.2, in our study. Although buffer solutions were added to cultures to avoid the influence of accumulated acid on pH (Grant and Mertens, 1992), we still observed some fluctuations in pH during culture incubation. Following the incubation period, our low pH cultures had a pH of 6.06 ± 0.01 and our high pH cultures had a pH of 6.21 ± 0.01. A recent in vitro study by Troegeler-

Meynadier et al. (2003 and 2013) also reported minor pH fluctuations (over 0.4 and 0.2 unit of changes on pH for low and high pH cultures, repectively). In our study, adding OIL did not affect the pH of cultures similar to Honkanen et al. (2012), who reported a similar result with no effect of linoleic acid on culture pH.

Previous in vitro studies utilized hydrochloride acid and sodium hydroxide solution to adjust pH of buffer solution (*e.g.* Lascano et al., 2016; Zened et al., 2011; Troegeler-Meynadier et al., 2003). However, this method might introduce excess salt into incubation cultures and influence the growth of bacteria. Citric acid can be metabolized by rumen bacteria (Van Soest, 1994), and was utilized in our study to avoid introducing negative effects on fermentation. The effectiveness of using citric acid and phosphoric acid to adjust pH of phosphate-bicarbonate buffer has previously been evaluated (Grant and Merten, 1992); compared with phosphoric acid, citric acid was better able to maintain pH in rumen cultures during 72 h of fermentation. In our study, approximately 0.14 and 0.27 g/culture of citric acid was added to flasks to achieve high pH and low pH cultures, respectively. Compared with phosphoric acid, using citric acid to adjust buffer pH had no negative effect on NDF digestion, and low pH decreased NDF digestion compared with high pH (Grant and Mertens, 1992). There are no studies, that we are aware of, that tested the effect of citric acid on BH. However, because rumen microbes are able to utilize citric acid as an energy source and produce acetic acid (Van Soest, 1994), we cannot rule out the effects of culture pH level on NDF digestion and BH being attributable to addition of citric acid.

BH extent of LA indicates the disappearance of LA during the incubation. Previous in vitro studies have reported higher BH extent of LA than our study in short-term incubation (< 8 h; Troegeler-Meynadier et al., 2003 and 2006; Zened et al., 2011; Honkanen et al., 2012). The difference may be resulted by several factors including: 1) higher culture pH (Troegeler-

Meynadier et al., 2003 and 2006; Honkanen et al., 2012), which increased isomerization of LA (Troegeler-Meynadier et al., 2006); 2) addition of free LA instead of triglyceride (Zened et al., 2011; Honkanen et al., 2012), which avoided the inhibition of low culture pH on hydrolysis of triglyceride (Van Nevel and Demeyer, 1996). Even though we observed lower BH extents after short-tem incubation (< 12 h), BH extents after 24 h of incubation were comparable with previous study (Troegeler-Meynadier et al., 2006). An in vivo study (Harvatine and Allen, 2006) reported over 80% BH extent of LA within approximately 12 h retention time in the rumen. In this study, other than relatively higher mean rumen pH (pH 6.0), the higher passage rate of BH intermediates from rumen might avoid the accumulation of intermediates of BH and resulted in greater isomerization of LA.

Previous studies have reported that low pH inhibits BH of LA, decreases *c*9, *t*11 CLA, and increases formation of *t*10, *c*12 CLA after no more than 8 h of short-term incubation (Fuentes et al., 2011; Troegeler-Meynadier et al., 2013). We also observed inhibition of low pH on BH of LA and accumulation of both *t*10, *c*12 CLA and *c*9, *t*11 CLA after 24 h of incubation. It is worth noting that the incubation times were only 6 h in previous studies (Van Nevel and Demeyer, 1996; Troegeler-Meynadier et al., 2013). In our 24-h incubation, *c*9, *t*11 CLA increased during the first 12 h and decreased during the second 12 h in high pH cultures containing OIL, but continued to increase in low pH cultures containing OIL across 24 h. Despite fluctuation, the ratio of *t*10, *c*12 CLA to *c*9, *t*11 CLA after 24 h of incubation were 56 and 35% for low and high pH cultures, respectively. Troegeler-Meynadier et al. (2003) also found low pH increased ratio of and *t*10, *c*12 CLA to *c*9, *t*11 CLA. Generally, cellulolytic bacteria are the major group of bacteria producing *c*9, *t*11 CLA and *t*11 18:1 during BH of LA (Polan et al., 1964). Cellulolytic bacteria are sensitive to rumen pH; when pH drops below 5.7,

cells of *Ruminococcus albus*, *Bacteroides succinogenes*, and *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens* stop growing in pure cultures (Russell and Dombrowski, 1980). However, *Megasphaera elsdenii*, one of the reported producers of *t*10, *c*12 CLA in vitro (Kim, et al., 2002), is more tolerant to low pH, and still grows in cultures until pH reaches 4.9 (Russell and Dombrowski, 1980). Therefore, it is more competitive than cellulolytic bacteria at low pH.

Trogegeler-Meynadier et al. (2006) reported that low pH inhibited isomerisation of LA into CLA intermediates, as well as the second reduction of *trans* 18:1 into saturated 18:0. We also observed lower BH extent of LA and formation of 18:0 in low pH compared to high pH cultures. *Butyrivibrio proteoclasticus*, the bacteria that hydrogenates *trans* 18:1 to 18:0, may also be sensitive to low pH (Wallace et al., 2006). Fuentes et al. (2009) reported that low pH decreased 18:0 but tended to increase *Butyrivibrio fibrisolvens* SA producer subgroup in the liquid of dual-flow continuous cultures. The authors proposed that *Butyrivibrio fibrisolvens* SA may play a minor role in the production of 18:0 or that metabolic activity of this species might not be proportional to its 16S rRNA concentration. Additionally, Boeckaert et al. (2009) found that compared with liquid-associated bacteria, accumulated more *trans* 18:1 intermediates, solid-associated bacteria biohydrogenate LA completely to 18:0, and *Butyrivibrio proteoclasticus* is more solid-associated bacteria, rather than flow in the rumen fluid (Boeckaert et al., 2009).

Dietary PUFA and alteration of rumen pH are two requirements for diet-induced MFD (Bauman and Griinari, 2003), and these factors interacted on the BH of LA in our study. Increasing OIL increased BH of LA in cultures with high pH. This suggests that bacteria might be able to increase BH capacity to compensate for increasing PUFA, or a minor increase in dietary PUFA might have no influence or stimulation on bacterial growth. Ivan et al. (2013) reported that 6% dietary LA-enriched oil increased total cellulolytic bacteria in rumen fluid. Van

Nevel and Demeyer (1996) tested effects of pH, from 5.2 to 6.8, on lipolysis and BH of 40 or 80 mg of soybean oil in vitro. This study suggested that 80 mg of soybean oil had greater inhibition on both lipolysis and BH than 40 mg of soybean oil at low culture pH. However, no substrate other than soybean oil was utilized in their incubation, which limited the nutrient supply and attachment site for rumen bacteria (Honkanen et al. 2012).

Increasing OIL resulted in more residual LA, and greater accumulation of *t*10, *c*12 CLA and *t*10 18:1 in cultures with low pH, compared to high pH. Previous studies found that *Megasphaera elsdenii* is low pH-tolerant and insensitive to PUFA (Russell and Dombrowski, 1980; Maia et al., 2007). The bacteria may be able to isomerize LA to *t*10, *c*12 CLA in culture, while growth and metabolism of other bacteria are inhibited by low pH and high PUFA. At high pH, OIL increased isomerisation of LA, but decreased hydrogenation of *trans* 18:1 to 18:0. This suggested that the bacteria associated with the last step of BH might be more sensitive to PUFA than bacteria associated with other steps of BH. Maia et al. (2007) observed similar results in a pure bacterial culture study, that butyrate-producing bacteria (eg. *Butyrivibrio proteoclasticus*) are more sensitive to UFA than other bacteria.

NDF disappearance extent was recorded to measure viability of cultures. Both culture pH and OIL affected NDF disappearance extent. In our study, compared with high pH, low pH decreased NDF disappearance extent by 35%. As previously mentioned, low rumen pH is detrimental to cellulolytic bacteria (Russell and Wilson, 1996). Grant and Mertens (1992) reported that both rate and lag time of NDF digestion were affected negatively when pH dropped below 6.2. Previous meta-analysis by Weld and Armentano (2015) showed that free oil supplementation decreased total tract NDF digestibility. Hristov et al. (2005) found that 5% dietary LA-rich oil decreased NDF digestibility of beef cattle. Interestingly, we observed an

increase in NDF disappearance extent with increasing OIL concentration. This may be explained by the fact that fiber-rich alfalfa hay was the sole substrate in our in vitro culture. Bateman and Jenkins (1998) reported that up to 8% of soybean oil could be added to a high fiber diet without depressing NDF digestibility. Also, it is noteworthy that the maximum OIL addition in our experiment was 2% of DM, which is lower than in other studies (Weld and Armentano, 2015; Hristov et al., 2005). While we did not measure the microbial biomass in this batch culture study, low pH and the addition of OIL decreased the concentration OBCFA, which suggests a reduction in microbial biomass. OBCFA, a group of FA mainly synthesized by rumen microbes, have previously been used as a marker for estimation of rumen microbial mass (Vlaeminck et al., 2005). However, increasing OIL also increased total FA content in cultures, and the difference in OBCFA content was less than 10% among cultures with different OIL concentrations. Effects of pH and OIL on BH and NDF disappearance extent could be the result of changes in total bacterial biomass and bacterial population, respectively. Culture pH had a greater influence on BH and NDF disappearance extent than OIL concentration. Martin and Jenkins (2002) also observed greater effects of culture pH on BH of UFA compared to other factors tested, including dilution rate and soluble carbohydrate concentration.

CONCLUSIONS

Low culture pH decreased both NDF disappearance extent and the BH extent of LA and increased the accumulation of biohydrogenation intermediates (total *trans* 18:1 and CLA). Especially, low pH resulted in great increase in both *t*10 18:1 and *t*10, *c*12 CLA. Addition of OIL increased NDF disappearance extent and BH extent of LA. Accumulation of both *t*10 18:1 and *t*10, *c*12 was greater with increasing OIL at low pH, compared to high pH. This implies a higher risk for MFD when diets contain high linoleic concentration, provided by vagetable oil, along

with factors that may cause low rumen pH, and it is more important to maintain a high rumen pH to reduce risk for MFD. Future studies will focus on the effects of other dietary factors on BH pathways, including starch content and fermentability, which often affect rumen pH, and some feed additives (*e.g.* yeast fermentation product), which have shown the function of alleviating MFD.

APPENDIX

APPENDIX

Table 2.1. FA profile and total FA content of alfalfa hay containing OIL used in in vitro batch culture.

	Alfalfa hay containing OIL[1]		
	0% OIL	1% OIL	2% OIL
FA, g/100 g total FA			
16:0	31.0	19.9	16.7
18:0	5.27	3.30	2.76
*c*9 18:1	4.37	17.9	21.8
LA[2]	18.4	39.0	44.8
*c*9, *c*12, *c*15 18:3	19.7	9.41	6.49
∑ Others	21.2	10.5	7.46
Total FA, % of DM	1.01	2.31	3.58

[1] Alfalfa hay contained 0% (0% OIL), 1% (1% OIL), or 2% corn oil (2% OIL).
[2] LA, linoleic acid (*c*9, *c*12 18:2).

Table 2.2. Effects of pH and OIL concentration on culture pH, total FA amount, LA biohydrogenation extent, and NDF disappearance extent after 24 h of incubation.[1]

Variable	Low pH			High pH			SEM	*P*-value[2]		
	0% OIL	1% OIL	2% OIL	0% OIL	1% OIL	2% OIL		pH	OIL	pH × OIL
pH	6.09	6.05	6.04	6.23	6.20	6.19	0.02	<0.001	NS	NS
NDF disappearance extent, %	19.1	22.7	26.3	31.7	35.2	37.8	0.7	<0.001	<0.001	NS
BH extent of LA[3], %	51.6	56.1	49.1	64.3	80.7	83.4	1.5	<0.001	0.001	0.001
Total FA, mg/culture	11.2	16.6	22.4	11.5	16.9	22.1	0.2	NS	<0.001	NS

[1] Values are means of 2 replicates for all variables, except pH value is the mean of 4 replicates.
[2] NS = not significant *(P* > 0.1).
[3] BH extent of LA, was calculated by subtracting the amount of LA in the cultures at 24 h from the amount at 0 h and dividing by the amount at 0 h.

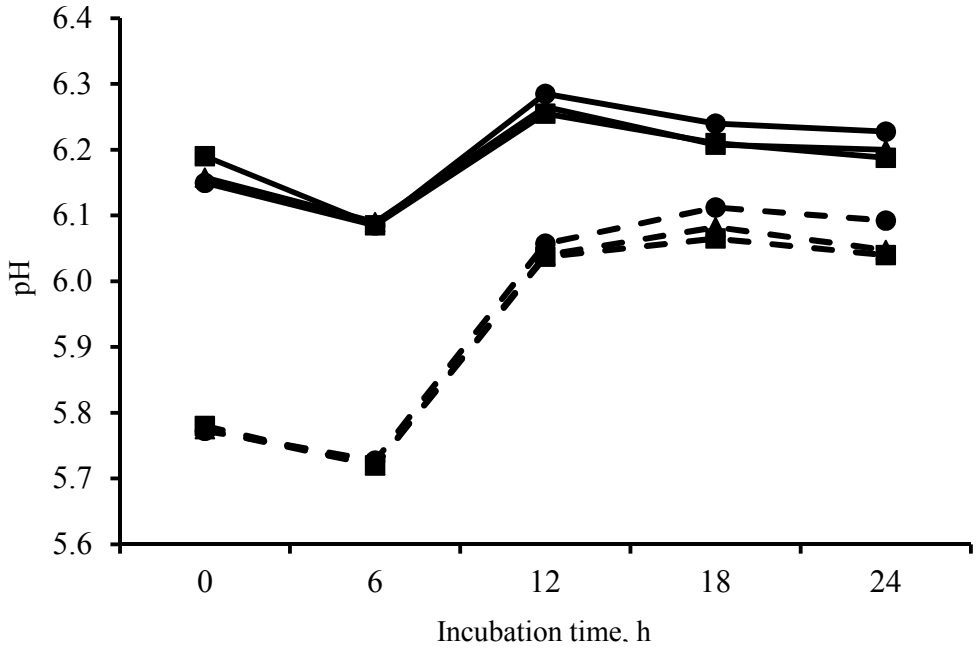

Figure 2.1. pH of cultures incubated with 0, 1, and 2 % OIL at low pH and high pH during 24 h of incubation.

pH of cultures incubated with 0 (③), 1 (⑩), and 2 % OIL (⑤) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.01; interaction between pH and time, $P < 0.001$; interaction between OIL and time, $P < 0.001$).

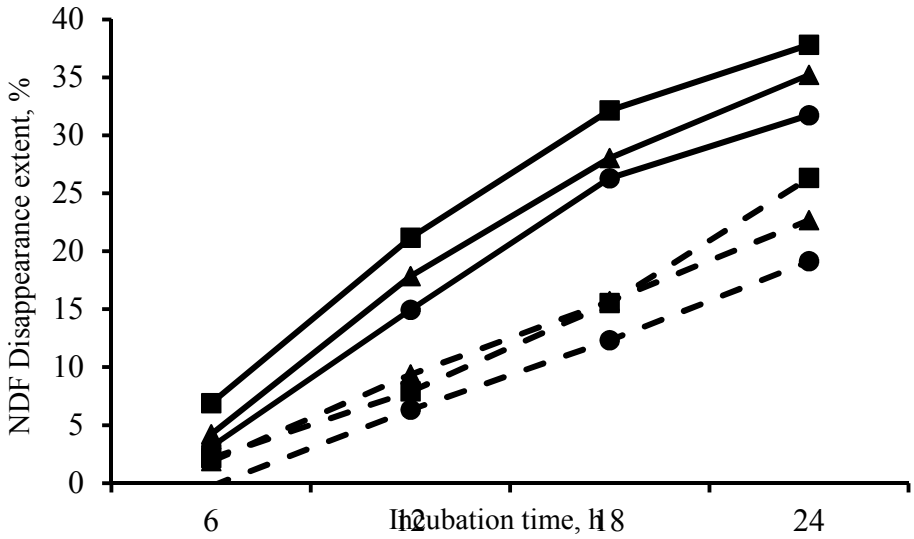

Figure 2.2. NDF disappearance extent in cultures incubated with 0, 1, and 2 % OIL at low pH and high pH during 24 h of incubation.
NDF disappearance extent in cultures incubated with 0 (③), 1 (⑩), and 2 % OIL (⑤) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.69; interaction among pH, OIL and time, $P = 0.06$). The NDF disappearance extent was calculated by subtracting the amount of NDF residue in the cultures at 6, 12, 18, and 24 h from the amount at 0 h and dividing by the amount at 0 h.

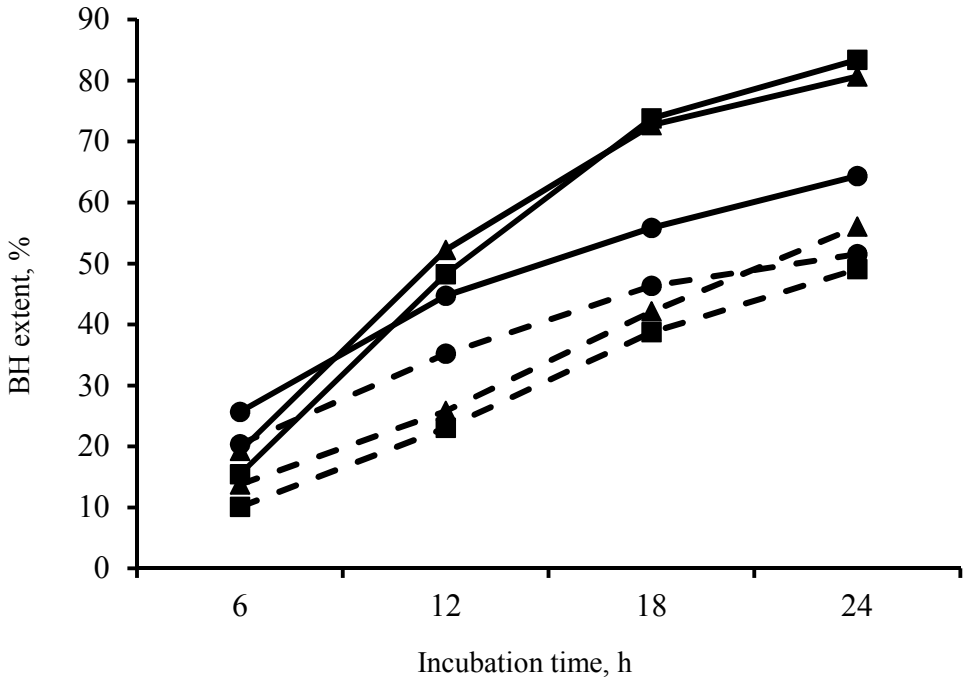

Figure 2.3. BH extent of LA in cultures incubated with 0, 1, and 2 % OIL at low pH and high pH during 24 h of incubation.

BH extent of LA (*c*9, *c*12 18:2) in cultures incubated with 0 (③), 1 (⑩), and 2 % OIL (⑤) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 1.03; interaction among pH, OIL and time, $P < 0.001$). The BH extent was calculated by subtracting the amount of LA in the cultures at 6, 12, 18, and 24 h from the amount at 0 h and dividing by the amount at 0 h.

Table 2.3. Effects of pH and OIL concentration on FA profile (g/100g total FA) of cultures after 24 h of incubation.[1]

FA (g/100 g)	Low pH			High pH			SEM	*P*-value[2]		
	0% OIL	1% OIL	2% OIL	0% OIL	1% OIL	2% OIL		pH	OIL	pH × OIL
12:0	0.92	0.61	0.46	0.81	0.53	0.41	0.01	<0.001	<0.001	0.08
iso 13:0	0.92	0.61	0.46	0.81	0.53	0.41	0.01	NS	<0.001	NS
13:0	0.18	0.12	0.09	0.22	0.14	0.11	0.003	<0.001	<0.001	0.08
14:0	2.30	1.56	1.16	2.72	1.79	1.32	0.03	<0.001	<0.001	0.01
iso 15:0	0.71	0.45	0.32	0.82	0.52	0.38	0.01	<0.001	<0.001	0.002
anteiso 15:0	2.86	1.92	1.38	3.44	2.21	1.64	0.03	<0.001	<0.001	0.004
15:0	1.66	1.17	0.82	2.29	1.55	1.15	0.02	<0.001	<0.001	0.001
iso 16:0	0.30	0.20	0.15	0.39	0.25	0.18	0.002	<0.001	<0.001	<0.001
16:0	23.7	19.4	17.4	23.9	19.4	17.5	0.07	NS	<0.001	NS
*c*7+*c*8 16:1	0.55	0.33	0.24	0.35	0.22	0.16	0.01	<0.001	<0.001	<0.001
*c*9 16:1	0.28	0.19	0.18	0.32	0.17	0.12	0.004	0.01	<0.001	<0.001
*c*10+*t*13 16:1	0.33	0.22	0.16	0.38	0.25	0.18	0.003	<0.001	<0.001	0.003
18:0	**28.1**	**20.3**	**15.3**	**32.3**	**32.0**	**28.6**	**0.73**	**<0.001**	**<0.001**	**0.002**
*t*4 18:1	0.04	0.03	0.03	0.03	0.06	0.07	0.005	0.001	0.05	0.01
*t*5 18:1	0.02	0.04	0.03	0.04	0.05	0.07	0.007	0.008	0.06	NS
*t*6 + *t*7 + *t*8 18:1	0.38	0.53	0.47	0.32	0.54	0.74	0.02	0.004	<0.001	<0.001
*t*9 18:1	0.32	0.57	0.61	0.25	0.48	0.69	0.007	0.007	<0.001	<0.001
***t*10, 18:1**	**1.00**	**1.86**	**2.27**	**0.57**	**0.97**	**1.33**	**0.05**	**<0.001**	**<0.001**	**0.003**
***t*11, 18:1**	**5.16**	**9.94**	**10.8**	**4.29**	**10.8**	**15.7**	**0.35**	**0.001**	**<0.001**	**<0.001**
*t*12 18:1	0.68	0.85	0.77	0.58	0.93	1.20	0.03	0.001	<0.001	<0.001
***c*9, 18:1**	**4.92**	**11.9**	**16.1**	**4.09**	**7.57**	**9.80**	**0.38**	**<0.001**	**<0.001**	**0.001**

Table 2.3. (cont'd)

FA (g/100 g)	Low pH			High pH			SEM	*P*-value[2]		
	0% OIL	1% OIL	2% OIL	0% OIL	1% OIL	2% OIL		pH	OIL	pH × OIL
*c*11 18:1	1.58	1.53	1.40	1.48	1.44	1.47	0.02	0.02	0.003	0.002
*c*12 18:1	0.73	0.77	0.69	0.64	0.76	0.89	0.02	0.06	0.003	<0.001
*c*13 18:1	0.06	0.05	0.04	0.05	0.06	0.05	0.004	NS	0.06	0.15
*c*14 + *t*16 18:1	0.40	0.28	0.18	0.48	0.51	0.47	0.01	<0.001	<0.001	0.001
*t*8, *t*10 + *t*9, *t*11 + *t*10, *t*12 18:2	0.19	0.31	0.45	0.11	0.27	0.39	0.09	NS	0.07	NS
***c*9, *t*11 CLA**	**0.07**	**0.29**	**0.39**	**0.06**	**0.23**	**0.33**	**0.01**	**0.004**	**<0.001**	**NS**
LA[3]	**5.61**	**12.4**	**18.3**	**4.07**	**5.12**	**5.95**	**0.51**	**<0.001**	**<0.001**	**<0.001**
*c*10, *c*12 CLA	0.09	0.06	0.04	0.08	0.06	0.05	0.003	NS	<0.001	NS
***t*10, *c*12 CLA**	**0.04**	**0.13**	**0.24**	**0.02**	**0.07**	**0.11**	**0.02**	**0.003**	**<0.001**	**0.04**
*c*11, *c*13 CLA	0.28	0.17	0.12	0.51	0.35	0.26	0.02	<0.001	<0.001	0.08
*t*11, *t*13 CLA	0.20	0.15	0.14	0.10	0.08	0.08	0.01	<0.001	0.003	0.06
***c*9, *c*12, *c*15 18:3**	**3.53**	**2.28**	**1.96**	**2.30**	**1.54**	**1.18**	**0.05**	**<0.001**	**<0.001**	**0.004**
19:0	0.52	0.38	0.29	0.47	0.33	0.27	0.01	<0.001	<0.001	NS
20:0	0.81	0.64	0.58	0.76	0.68	0.63	0.004	0.004	<0.001	<0.001
*c*11 20:1	0.11	0.15	0.19	0.09	0.12	0.14	0.01	<0.001	<0.001	NS
*c*11, *c*14 20:2	0.07	0.05	0.05	0.05	0.04	0.04	0.003	<0.001	0.002	NS
*c*5, *c*8, *c*11 20:3	0.04	0.02	0.03	0.02	0.02	0.01	0.002	<0.001	0.005	NS
22:0	0.84	0.55	0.44	0.75	0.61	0.50	0.02	NS	<0.001	0.004
23:0	0.16	0.12	0.09	0.19	0.14	0.10	0.004	<0.001	<0.001	0.07
24:0	0.71	0.45	0.38	0.59	0.56	0.47	0.04	NS	0.005	0.07

Table 2.3. (cont'd)

FA (g/100 g)	Low pH			High pH			SEM	*P*-value[2]		
	0% OIL	1% OIL	2% OIL	0% OIL	1% OIL	2% OIL		pH	OIL	pH × OIL
Unknown	9.28	6.82	5.29	8.75	6.45	5.14	0.14	0.02	<0.001	NS
Total *trans* 18:1	7.61	13.8	14.9	6.09	13.8	19.8	0.42	0.017	<0.001	<0.001
SFA[4]	59.8	45.3	37.0	65.0	57.7	51.0	0.70	<0.001	<0.001	0.002
MUFA *cis* [5]	8.96	15.4	19.1	7.89	11.1	13.3	0.39	<0.001	<0.001	0.002
PUFA *cis* [6]	9.25	14.7	20.3	6.45	6.71	7.19	0.55	<0.001	<0.001	<0.001
BCFA[7]	4.21	2.79	2.00	4.99	3.20	2.36	0.04	<0.001	<0.001	0.003
OCFA[8]	2.52	1.79	1.30	3.17	2.16	1.63	0.03	<0.001	<0.001	0.003
OBCFA[9]	6.73	4.58	3.29	8.15	5.36	3.99	0.06	<0.001	<0.001	0.002

[1] Values are means of 2 replicates for all variables.
[2] NS = not significant ($P > 0.1$).
[3] LA, linoleic acid (*c*9, *c*12 18:2).
[4] SFA: saturated FA; [5] MUFA *cis*: monounsaturated FA; [6] PUFA *cis*: polyunsaturated FA; [7] BCFA: branched-chain FA. [8] OCFA: odd-chain FA; [9] OBCFA: odd- and branched-chain FA

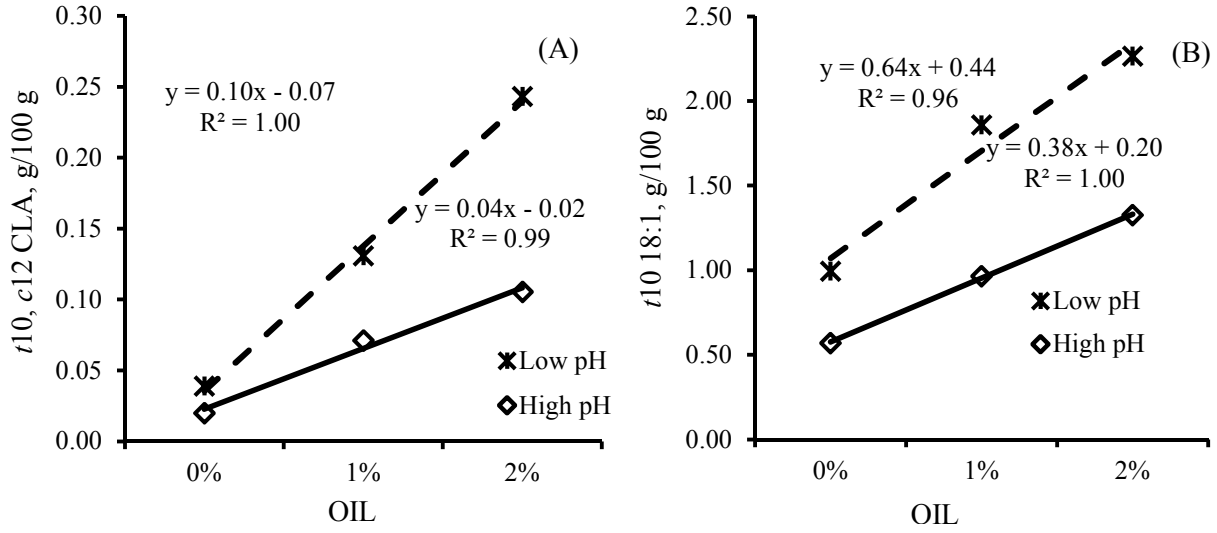

Figure 2.4. Interaction between pH and OIL concentration and its effect on concentrations of *t*10, *c*12 CLA (A) and *t*10 18:1 (B) in culture after 24 h of incubation. *P* = 0.04 and 0.003, respectively.

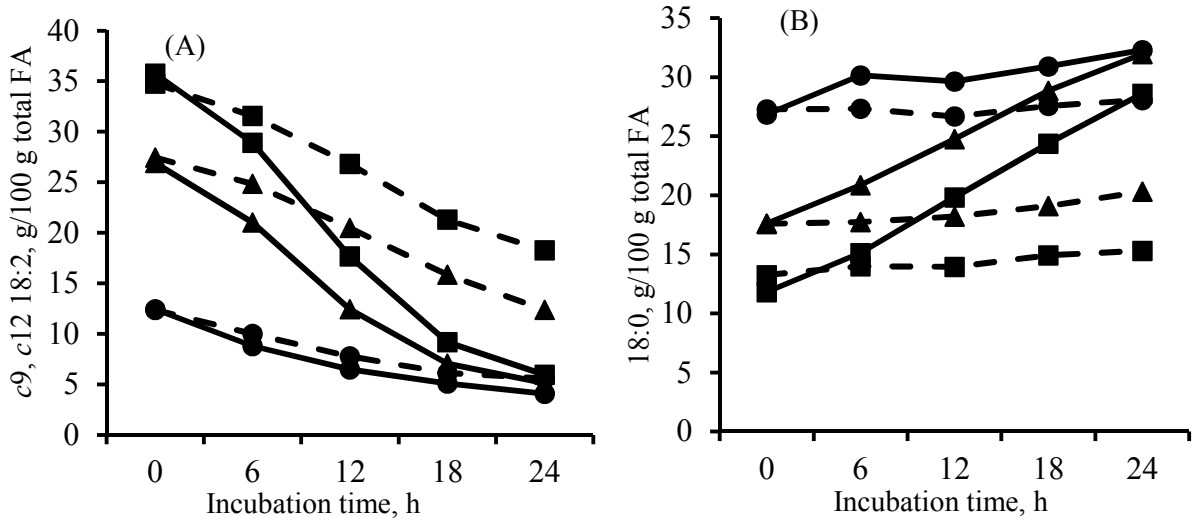

Figure 2.5. Concentrations of LA (A) and 18:0 (B) in cultures incubated with 0, 1, and 2 % OIL at low pH and high pH during 24 h of incubation.
Concentrations of LA (*c*9, *c*12 18:2; A) and 18:0 (B) in cultures incubated with 0 (③), 1 (⑩), and 2 % OIL (⑤) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.32 and 0.43, respectively; interaction among pH, OIL and time, both $P < 0.001$).

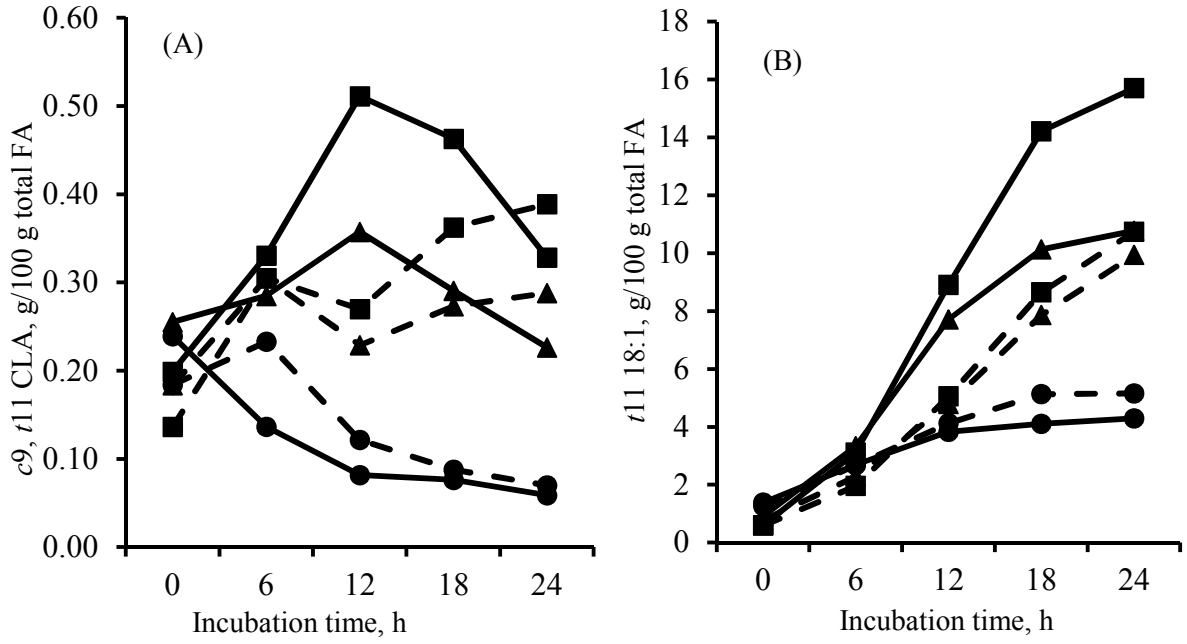

Figure 2.6. Concentrations of *c*9, *t*11 CLA (A) and *t*11 18:1 (B) in cultures incubated with 0, 1, and 2 % OIL at low pH and high pH during 24 h of incubation.
Concentrations of *c*9, *t*11 CLA (A) and *t*11 18:1 (B) in cultures incubated with 0 (③), 1 (⑩), and 2 % OIL (⑤) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.01 and 0.20, respectively; interaction among pH, OIL and time, both $P < 0.001$).

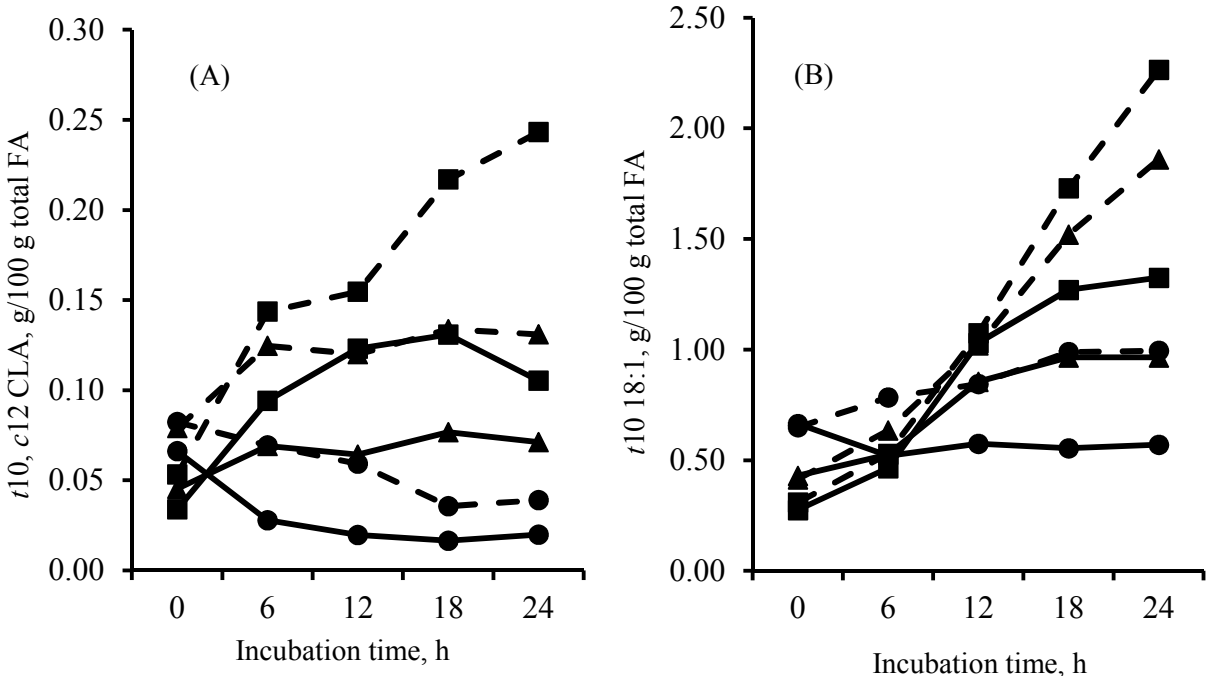

Figure 2.7. Concentrations of *t*10, *c*12 CLA (A) and *t*10 18:1 (B) in cultures incubated with 0, 1, and 2 % OIL at low pH and high pH during 24 h of incubation.
Concentrations of *t*10, *c*12 CLA (A) and *t*10 18:1 (B) in cultures incubated with 0 (③), 1 (⑩), and 2 % OIL (⑤) at low pH (dashed line) and high pH (solid line) during 24 h of incubation (SEM = 0.01 and 0.03, respectively; interaction among pH, OIL and time, both $P < 0.01$).

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CHAPTER 3

EFFECTS OF STARCH FERMENTABILITY, STARCH CONTENT, AND CULTURE PH ON THE BIOHYDROGENATION OF UNSATURATED FATTY ACIDS IN BATCH CULTURE

INTRODUCTION

Milk fat depression (MFD) has challenged both dairy producers and nutritionists for over a century. Past research shows that MFD is caused mainly by the inhibition of milk fat synthesis by specific FA (*e.g.* *t*10, *c*12 conjugated linoleic acid, CLA) produced during biohydrogenation (BH) of dietary unsaturated FA (UFA) in the rumen. BH is the process that rumen bacteria saturate UFA and produce various FA intermediates (Shingfield and Wallace, 2014). Several dietary and ruminal factors can influence BH and increase the formation of MFD-associated BH intermediates, thus increasing the risk of MFD.

Diet-induced MFD is commonly seen when dairy cow diets contain large amounts of highly fermentable carbohydrate, unsaturated oil (Bauman et al., 2011), or both. Diets high in starch typically reduce rumen pH due to increased fermentation acid production, decreased rumen VFA absorption, and decreased salivary buffer secretion (Allen, 1997). Rumen pH is positively correlated with milk fat content (Allen, 1997), and low rumen pH is known to promote BH pathways that increase formation of MFD-associated FA intermediates (Fuentes et al., 2011; Sun et al., 2014). We previously found that increasing corn oil at low pH, in vitro, resulted in greater formation of *t*10, *c*12 CLA than at high pH (Sun et al., 2014). Oba and Allen (2003) reported decreased milk fat concentration in cows fed high moisture corn (HMC) in a high-starch diet, but not in a low-starch diet. However, the high-starch diet also decreased rumen pH in the same study. It is not clear if reduced rumen pH or high starch fermentability and content was the driver for MFD. Fuentes et al. (2009) tested the effect of concentrate level and pH on BH in a

dual-flow continuous culture, and found that high level of concentrate increased accumulation of *t*10, *c*12 CLA by reducing pH. Lascano et al. (2016) tested the effect of starch degradability on BH in continuous cultures, and observed that highly degradable starch increased formation of *t*10, *c*12 CLA. However, this study only tested one level of starch content (around 21%), which was lower than the content in common commercial diets.

In vitro systems (batch culture and continuous culture) are commonly used to test the effects of dietary and ruminal factors on fermentation and microbial metabolism (Fuentes et al., 2009; Zened et al., 2011; Vlaeminck et al., 2008). We previously developed an in vitro batch culture system to test the effect of increasing corn oil on BH pathways at two different pH levels (Sun et al., 2014). The incubation and FA analysis were completed in the same culture tube to allow for accurate and precise FA analysis results. Therefore, the objective of our study was to determine the impact of starch fermentation and starch content on BH pathways at two pH levels. We hypothesized that both high starch fermentability and high starch content would increase the formation of MFD-associated BH intermediates, and the increase would be greatest at low culture pH.

MATERIALS AND METHODS

Treatment and Incubation

This experiment utilized in vitro batch culture system methodology as described in our previous study (Sun et al., 2014). Batch cultures were run in a randomized design with a $2 \times 2 \times 2$ factorial arrangement of treatments: two in vitro culture pH levels (low pH, 5.8 or high pH, 6.2), two starch sources (dry corn [DC] or high moisture corn [HMC]), and two starch concentrations (low starch, 22% or high starch, 33%). All cultures were run in quadruplicate. Culture substrate contained (DM basis) 70 or 55% alfalfa hay as forage source, and 30 or 45%

DC or HMC as starch sources to provide 22 or 33% starch (Table 3.1). Corn oil was added at 2% DM to alfalfa hay to increase the total UFA content of the substrates. Corn grain and alfalfa hay were dried at 55°C with a forced-air oven, and were ground through a 1-mm screen of an abrasion mill (UDY Corp., Fort Collins, Colorado) and through a 1-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA), respectively. Total FA content and FA profile of substrates are shown in Table 3.1.

All cultures were prepared using a modification of the method developed by Goering and Van Soest (1970). Pyrex centrifuge tubes (100 mL) were used for culture incubation, and contained: 500 mg of substrate (Table 3.1), 40 mL of buffer medium, 2 mL of reducing solution, and 10 mL of strained rumen inoculum. Precise substrate weights were recorded for each culture tube. Rumen fluid and digesta were collected through rumen cannula from three rumen-fistulated mid-lactation Holstein dairy cows (107 ± 98 DIM) fed a consistent TMR (27% corn silage, 14% haylage, 20% ground corn, 17% soybean meal, 7% high-moisture corn, 2% soy hulls, 3% wheat straw, 7% cottonseed, 3% mineral-vitamin). After collection, rumen fluid and digesta were mixed in equal proportions and transferred into a pre-warmed Thermos container. To detach bacteria from digesta particles, rumen fluid and digesta were blended for 15 s in a 1-gallon Waring blender. The mixture was passed through a Buchner funnel lined with nylon mesh and glass wool to filter out particles, and the strained fluid was used as inoculum for in vitro batch culture incubation. After adding rumen inoculum, culture tubes were flushed with CO_2 and sealed with 5.5-cm rubber stoppers connected to a Bunsen valve by a glass tube. Temperature of all cultures was maintained at 39°C in a water bath during incubation. Citric acid solution (1 M) was used to adjust buffer medium pH to 5.8 or 6.2 as described by Grant and Mertens (1992).

Incubation of sampled cultures was stopped at 0, 12 and 24 h of incubation. At sampling time, the pH of all four replicates was measured with a pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland). Incubations were terminated after pH measurement by adding dry ice cubes into culture tubes and placing culture tubes in an ice batch. Culture tubes for NDF residue analysis (2 replicates) were placed in a 4°C cooler, and the analysis was completed within 48 h. Culture tubes for FA composition analysis (2 replicates) were stored at -20°C, and the freeze-drying process was completed directly in the culture tubes.

Sample Analysis

NDF residue in cultures was analyzed as described by Mertens (2002). FA in cultures were methylated directly in the culture tubes after freeze-drying using a 2-step methylation protocol adapted from Jenkins (2010) as described by Sun et al. (2014). Heptadecanoic acid (17:0, 1:1 mg/mL toluene) was used as an internal standard and was added to culture tubes after the termination of incubations and prior to storage at -20°C. Culture content DM weight was determined by subtracting weights of empty tubes from weights of tubes containing cultures after freeze-drying. Quantification of FA composition including approximately 45 FA in the range of 12:0 to 24:0 was determined using a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with a split injector (1:100 split ratio) and a flame-ionization detector (FID) using a CP-Sill 88 WCOT (wall-coated open tubular) fused-silica column (100 m × 0.25 mm i.d. × 0.2-μm film thickness; Varian Inc., Lake Forest, CA) as described by Sun et al. (manuscript in preparation).

Calculations and Statistical Analysis

NDF disappearance extent was calculated by subtracting the content of NDF in the cultures at 12 and 24 h from the content at 0 h, and dividing by the content at 0 h. The extent of

BH for *c*9, *c*12 18:2 (LA) was calculated by subtracting the content of LA in the cultures at 12 and 24 h from the content at 0 h, and dividing by the content at 0 h. Appearance rates of individual FA were calculated for *t*10, *c*12 CLA and *t*10 18:1 as follows: FA concentration increase in each 12-h incubation period (0 to 12 h or 12 to 24 h) divided by 12 h.

All data from in vitro batch cultures were analyzed with the fit model procedure of JMP (Version 10, SAS Institute). The pH, NDF disappearance extent, extent of BH, total FA content, and FA concentrations of in vitro batch cultures after 24 h of incubation were analyzed using a model including main effects of in vitro culture pH level, starch source, starch content, and the interactions of main effects. In addition, to test the treatment effects across time on NDF disappearance extent, extent of BH, *t*10, *c*12 CLA, and *t*10 18:1 concentrations, results obtained at each sampling time (0, 12, and 24 h of incubation) were analyzed separately using the same model but with the effect of time and interactions with main effects and their interactions included. Interactions were removed from the model if $P > 0.15$. Least square means with standard error are reported, and significance declared at $P < 0.05$.

RESULTS

FA content and profile of substrates

Total FA content and FA profile were consistent for substrates containing DC and HMC (Table 3.1). There were minor differences in FA profile between substrates with low and high starch content. The major FA in all substrates was LA, which comprised close to 50% of total FA.

pH, NDF disappearance extent, and BH extent of in vitro cultures

Effects of treatment on pH, NDF disappearance extent, total FA content, and BH extent of LA of cultures after 24 h of incubation are shown in Table 3.2. Changes in pH, NDF

disappearance extent, and BH extent of LA are shown in Figures 3.1-3.3 Overall, pH of all cultures decreased throughout incubation, and especially in low pH cultures (5.80 to 5.78) compared with high pH cultures (6.23 to 6.00) from 0 to 24 h of incubation (Figure 3.1). After 12 h of incubation, cultures with HMC had lower pH than those containing DC (5.86 vs. 5.88, $P < 0.01$); however, after 24 h of incubation, cultures with HMC had higher pH than DC (5.91 vs. 5.87, $P < 0.001$). Compared with low starch cultures, high starch cultures decreased pH throughout the incubation (5.81 vs. 5.93 and 5.84 vs. 5.94, respectively; both $P < 0.001$). However, the mean pH of low and high pH cultures were 5.78 and 6.00, respectively ($P < 0.001$), and the difference between low and high pH cultures was above 0.2 units across time. Overall, low pH increased total FA content compared with high pH (23.0 vs. 22.3 mg/culture, $P < 0.001$). Culture pH interacted with starch content and starch fermentability on total FA content ($P < 0.05$). Compared with low starch cultures, high starch cultures increased total FA content at low pH ($P < 0.05$), but had no effect at high pH. HMC did not affect total FA content at low pH compared with DC, but decreased total FA content at high culture pH ($P < 0.05$).

Overall, after 24 h of incubation, low pH decreased NDF disappearance extent (11.0 vs. 28.7%, $P < 0.001$) and BH extent (29.4 vs. 61.5%, $P < 0.001$) compared with high pH (Table 3.2). High starch decreased NDF disappearance extent (18.1 vs. 21.6%, $P < 0.001$) and BH extent (42.7 vs. 48.3%, $P < 0.001$) compared with low starch. Compared with DC, HMC decreased NDF disappearance extent (18.5 vs. 21.2%, $P < 0.01$). Starch fermentability interacted with starch content on NDF disappearance extent ($P < 0.05$), with no difference between DC and HMC in low starch cultures, but HMC decreased NDF disappearance extent in high starch cultures compared with DC (15.6 vs. 20.5%, $P < 0.001$). We also observed a three-way interaction among starch fermentability, starch content, and culture pH, in which HMC

decreased NDF disappearance extent compared with DC in high starch cultures at high culture pH (22.7 vs. 30.0%, $P < 0.001$). Overall, starch fermentability had no effect on BH extent. Culture pH interacted with starch content and starch fermentability on BH extent respectively (both $P < 0.001$). Compared with high starch cultures, low starch cultures increased BH extent by 7.5% at low culture pH (30.5 vs. 28.4%, $P < 0.05$), and by 15.8% at high culture pH (66.0 vs. 57.0%, $P < 0.001$). Compared with DC, HMC increased BH extent at low culture pH (31.8 vs. 27.0%, $P < 0.001$), but decreased BH extent at high culture pH (58.0 vs. 65.1%, $P < 0.001$).

Changes in NDF disappearance extent and BH extent of LA during the 24-h incubation period are shown in Figures 3.2 and 3.3, respectively. Compared to low pH, high pH increased both NDF disappearance extent (1.20 vs. 0.53%/h) and BH extent (1.75 vs. 1.14%/h) at greater rates from 12 to 24 h of incubation (Table 3.3). Cultures increased NDF disappearance extent at similar increasing rates at low pH; however, at high pH, low starch cultures with HMC had a faster increasing rate than other cultures (Table 3.3). Low starch cultures with HMC increased BH extent faster than other cultures at low pH; while high starch cultures with HMC increased BH extent slower than other cultures at high pH (Table 3.3).

Effects of culture pH, starch content, and starch fermentability on FA profile of cultures after 24 h of incubation

Table 3.4 shows the effects of culture pH, starch content, and starch fermentability on FA profile of cultures after 24 h of incubation. Overall, compared to high pH, low pH decreased total conjugated linoleic acid (CLA; 0.93 vs. 1.36 g/100 g, $P < 0.001$), total *trans* 18:1 FA (10.5 vs. 16.8 g/100g, $P < 0.001$), and total odd- and branched- chain FA (OBCFA; 2.79 vs. 3.81 g/100g, $P < 0.001$). Compared to high pH, low pH increased LA (25.8 vs. 14.4 g/100 g, $P < 0.001$), *t*10, *c*12 CLA (0.29 vs. 0.22 g/100 g, $P < 0.001$), and *t*10 18:1 (3.10 vs. 1.90 g/100 g, $P < 0.001$), but

decreased *c*9, *t*11 CLA (0.17 vs. 0.45 g/100 g, $P < 0.001$), *t*11 18:1 (6.4 vs. 12.5 g/100 g, $P < 0.001$), and 18:0 (13.0 vs. 19.6 g/100 g, $P < 0.001$).

Compared with low starch cultures, high starch cultures exhibited decreased total *trans* 18:1 FA (13.1 vs. 14.2 g/100g, $P = 0.001$) and total OBCFA (3.25 vs. 3.34 g/100g, $P < 0.05$). Starch content had no effect on total CLA and *c*9, *t*11 CLA. Compared with low starch cultures, high starch cultures exhibited increased LA (21.4 vs. 18.8 g/100 g, $P < 0.001$), *t*10, *c*12 CLA (0.28 vs. 0.24 g/100 g, $P < 0.001$), and *t*10 18:1 (2.71 vs. 2.29 g/100 g, $P < 0.001$), and decreased *t*11 18:1 (8.80 vs. 10.2 g/100 g, $P < 0.001$) and 18:0 (15.6 vs. 17.1 g/100g, $P < 0.001$). Compared with DC, HMC decreased total CLA (0.96 vs. 1.33 g/100 g, $P < 0.001$) and total OBCFA (3.19 vs. 3.40 g/100 g, $P < 0.001$), and increased total *trans* 18:1 FA (14.3 vs. 13.0 g/100g, $P < 0.001$). Starch fermentability had no effect on LA. Compared with DC, HMC decreased *t*10, *c*12 CLA (0.23 vs. 0.28 g/100 g, $P < 0.001$), *c*9, *t*11 CLA (0.24 vs. 0.38 g/100g, $P < 0.001$), and 18:0 (15.3 vs. 17.4 g/100 g, $P < 0.001$), and increased *t*10 18:1 (2.89 vs. 2.11 g/100 g, $P < 0.001$) and *t*11 18:1 (9.78 vs. 9.21 g/100 g, $P < 0.05$).

Culture pH interacted with starch content to influence *t*10, *c*12 CLA concentration ($P < 0.05$). At low pH, high starch increased *t*10, *c*12 CLA compared with low starch (0.33 vs. 0.26 g/100g, $P < 0.01$), but there was no difference at high pH. We also observed a three-way interaction among culture pH, starch content, and starch fermentability ($P < 0.001$). At low pH, low starch cultures with HMC decreased *t*10, *c*12 CLA compared with DC (0.21 vs. 0.31 g/100 g, $P < 0.001$); at high pH, high starch with HMC treatment decreased *t*10, *c*12 CLA compared with DC (0.18 vs. 0.27 g/100 g, $P < 0.01$). Culture pH interacted with starch fermentability to influence *t*10 18:1 concentration ($P < 0.001$). Compared with DC, HMC increased *t*10 18:1 by

48 and 20% at low (3.71 vs. 2.50 g/100 g, $P < 0.001$) and high pH (2.07 vs. 1.73 g/100 g, $P < 0.01$), respectively.

Changes in t10, c12 CLA and t10 18:1 concentrations in culture over 24 h of incubation

The effects of treatment across time on *t*10, *c*12 CLA and *t*10 18:1 are shown in Figures 3.4 and 3.5, respectively. The appearance rates of *t*10, *c*12 CLA and *t*10 18:1 from 0 to 12 h and 12 to 24 h incubation are shown in Table 3.5. Overall, the appearance rate of *t*10, *c*12 CLA was higher during the first 12 h than the second 12 h of incubation (0.015 vs. 0.003 g/100 g/h; Figure 3.4). From 0 to 12 h, cultures containing HMC exhibited a greater appearance rate and higher concentration of *t*10, *c*12 CLA (0.42 vs. 0.24 g/100 g) than cultures containing DC at low pH (Table 3.5); however, from 12 to 24 h of incubation, HMC exhibited a decreasing concentration of *t*10, *c*12 CLA, which led to a negative appearance rate at low culture pH. At high pH, concentration of *t*10, *c*12 CLA increased throughout the 24-h incubation. Compared with DC, high starch cultures containing HMC exhibited a lower appearance rate at high pH and the lowest *t*10, *c*12 CLA concentration after 24 h of incubation. The appearance rate of *t*10 18:1 was higher during the second 12 h than the first 12 h of incubation (0.116 vs. 0.071 g/100 g/h; Figure 3.5). At both low and high pH, cultures containing HMC showed a higher appearance rate for *t*10 18:1, than those containing DC, across starch content levels. From 12 to 24 h, high starch cultures containing HMC showed *t*10 18:1 increasing at a faster rate than in other cultures across pH levels.

DISCUSSION

Dietary UFA are biohydrogenated by rumen bacteria across several steps, producing various FA intermediates (Griinari and Bauman, 1999). Linoleic acid (*c*9, *c*12 18:2) is one of the major UFA in dairy cows diet, and was also the most abundant FA in the substrates used in our

current study. During BH, LA is converted to CLA by isomerisation, and then converted to *trans* 18:1 FA and 18:0 by two hydrogenation steps. Normally, *c*9, *t*11 CLA and *t*11 18:1 make up the major CLA and *trans* 18:1 FA intermediates formed. However, specific dietary and rumen environmental changes can shift BH, thus increasing the formation of *t*10, *c*12 CLA and *t*10 18:1 by an alternative pathway. It has been well established that *t*10, *c*12 CLA is a potent milk fat synthesis inhibitor, and *t*10 18:1 is often used as a robust marker of MFD (Bauman et al., 2011).

Oba and Allen (2003) reported that feeding cows HMC in a high-starch diet led to lower milk fat concentration compared with DC in a similar diet; however, starch source did not influence milk fat concentration when fed in a low-starch diet. It is worth noting that a high-starch diet also reduced rumen pH in the same study, the MFD-inducing effect of HMC in a high starch diet might be caused by a reduction in rumen pH, rather than HMC itself. Based on our previous study (Sun et al. 2014), pH had the greatest effect on BH pathways of UFA, and concentration of *t*10, *c*12 CLA. Previously, we successfully utilized in vitro batch culture methodology to determine the interaction between culture pH and corn oil concentration and its effect on UFA BH (Sun et al., 2014). Therefore, the objective of our current study was to examine effects of fermentability characteristics at two starch content levels on UFA BH at low and high culture pH using in vitro batch culture. Although we selected two starch sources with different fermentabilities and utilized low and high starch levels in the substrates, total FA content and major FA concentrations did not differ between substrates.

Several researchers have previously varied pH, in vitro, from 5.6 to 6.78, to discern the effect of this variable on BH (Calsamiglia et al., 2002; AbuGhazaleh et al., 2005; Fuentes et al., 2011; Troegeler-Meynadier et al., 2013). In the current study, we chose a pH of 5.8 as the initial low pH due to known inhibition of rumen bacteria growth and nutrient digestibility at this level

(Russell and Dombrowski, 1980; Russell and Wilson, 1996), and it has been used as a threshold value to indicate subclinical ruminal acidosis in previous studies (Beauchemin and Yang, 2005; Dohme et al., 2008; Mohammed et al., 2012). A pH of 6.2 was selected to represent high pH. As described in Chapter 2, approximately 0.27 or 0.14 g/culture of citric acid was added into cultures to obtain low and high pH. Although citric acid had no negative effect on NDF digestion (Grant and Merten, 1992), we can not rule out the effects of pH level on NDF digestion and BH being attributable to addition of citric acid, due to the fact of citric acid can be energy source for rumen microbes (Van Soest, 1994).

Although buffer solution is widely used in in vitro studies to prevent acid accumulation and dramatic pH changes (Grant and Mertens, 1992), pH fluctuation is often observed during incubation (Troegeler-Meynadier et al., 2013; Sun et al., 2014). In the current study, the overall pH fluctuation exhibited different patterns in cultures started at low and high pH, in which low pH cultures increased pH but high pH cultures decreased pH from 12 to 24 h of incubation. Lactic acid-utilizing bacteria, such as *Megasphaera elsdenii* and *Selenomonas ruminantium*, have a higher tolerance of low pH according to Russell and Dombrowski (1980). Especially, *Megasphaera elsdenii* have maximum growth and highest growth rate at pH 6.0 (Therion et al., 1982). These lactic acid-utilizing bacteria may have utilized the lactic acid produced during incubation, and subsequently raised pH to a greater extent in low pH cultures. Hession and Kung (1995) investigated the effect of *Megasphaera elsdenii* inoculation on lactic acid accumulation and found that *Megasphaera elsdenii* could effectively prevent lactic acid accumulation and pH reduction. We also found that a high level of starch substrate decreased culture pH. It is common to observe reduction in rumen pH with a high starch diet due to increased production of fermentation acid, lower rumen absorption rates of VFA, and lower salivary buffer secretion

(Allen, 1997). Also, high starch diets are known to increase the growth rate of lactate-producing bacteria, such as *Streptococcus bovis*, which utilize glucose, produce lactate, and decrease rumen pH (Russell and Hino, 1985). Interestingly, we observed that HMC increased pH after 24 h of incubation, especially in cultures with high starch at high pH. It is a common belief that increasing dietary carbohydrate fermentability causes rumen pH reduction (Krause and Combs, 2003; Chibisa et al., 2015). However, Broderick et al. (2008) reported no effect on rumen pH when replacing dietary starch with sucrose from 0 to 7.5% DM, and Martel at el. (Martel et al., 2011) observed that 5% dietary molasses increased rumen pH from 5.73 to 5.87. Potential explanations were proposed by researchers: 1) highly fermentable carbohydrate increased production of butyrate, which had higher absorption rate by rumen epithelium than other VFA (Martel et al., 2011), or 2) highly fermentable carbohydrate increased the population of rumen microbes (Herrera-Saldana et al., 1990). Due to the closed nature of in vitro culture systems, the potential effect of highly fermentable carbohydrate on absorption of VFA is excluded. Lascano et al. (2016) also found that increasing starch fermentability was associated with increased pH in continuous fermenters. In the current study, it is reasonable to assume that HMC increased microbial N, or the specific microbes utilizing fermentable carbohydrate as an energy source. It was not our objective to test the microbial population in current study. However, we were able to indirectly obtain information about the microbial population from NDF disappearance extent, which represents the viability of cultures, and OBCFA, which is used as a marker for estimation of rumen microbial mass (Vlaeminck et al., 2005).

Several studies have reported the effect of pH on microbial metabolism and BH of UFA during various incubation times (Russell and Dombrowski, 1980; Kim, et al., 2002; Fuentes et al., 2011; Troegeler-Meynadier et al., 2013; Sun et al., 2014). By selecting a narrower pH range (5.8

vs. 6.2) and longer incubation time (24 h) than other studies, our group previously tested the interaction between culture pH and corn oil concentration on NDF disappearance extent and BH (Sun et al., 2014). The effect of pH was well discussed in that manuscript. In the current study, we focus on discussing the effect of starch content and starch fermentability on BH, and their interactions with culture pH.

Gerson et al. (1984) conducted short term (≤ 2 h) in vitro culture studies to test the effects of carbohydrate fermentability and content on lipolysis and hydrogenation by using rumen digesta from sheep fed different diets. They found that increasing starch content in the diet decreased both lipolysis and the BH rate. Fuentes et al. (2009) investigated effects of concentrate level and pH on BH in a dual-flow continuous culture and found that a high concentrate level increased lipolytic bacteria, such as *Anaerovibrio lipolytica*, DNA concentration at high pH, but had no effect on tested BH bacteria. We observed that high starch content decreased BH extent of linoleic acid, and high starch content combined with low pH led to the lowest BH extent. Correspondingly, high starch content decreased pH and high starch content at low pH led to the lowest pH after 24 of incubation. Rumen bacteria, especially cellulolytic bacteria, are highly sensitive to pH changes and are essential for BH (Russell and Dombrowski, 1980; Polan et al., 1964). We previously reported that low pH negatively affected BH (Sun et al., 2014). Therefore, the effect of starch content on BH extent might be partially mediated through pH reduction. Zened et al. (2012) tested the effect of starch level on BH and also found that cultures at low pH exhibited decreased BH extent. Similarly, the interaction between starch fermentability and culture pH on BH extent, which HMC increased BH extent at low pH and decreased BH extent at high pH compared with DC, could be partly explained by pH. BH extents were over 40% for high starch culture supplemented with LA after 5 h of incubation in studies of Zened et al., (2011

and 2012), which were relatively higher than our results after 12 h of incubation. As we previously discussed in Chapter 2, the difference might be caused by higher culture pH and addition of free LA.

*T*10, *c*12 CLA is a potent milk fat synthesis inhibitor produced by an alternative BH pathway and *t*10 18:1 is hydrogenated from *t*10, *c*12 CLA (Bauman et al., 2011). Fuentes (2009) observed that, from 1 to 24 h after feeding, *t*10, *c*12 CLA increased at pH 6.4, but decreased at pH 5.6. We also observed a similar pattern for *t*10, *c*12 CLA at low and high pH during incubation period. Overall, high starch content increased *t*10, *c*12 CLA, and the changes in *t*10, *c*12 CLA concentration followed the pattern of pH changes, in which low pH was associated with high *t*10, *c*12 CLA and high pH was associated with low *t*10, *c*12 CLA. Additionally, our NDF disappearance extent and OBCFA results were consistent with the effect of high starch on pH. Calsamiglia et al. (2009) tested the effects of diet type and pH on rumen microbial fermentation and found that pH, not diet, was the main factor influencing organic matter and NDF digestion. Therefore, high starch content might influence BH and *t*10, *c*12 CLA through pH. Fuentes et al. (2009) also reported that the effect of increased concentrate on BH was mainly caused by associated pH changes rather than the concentrate, itself.

The effects of starch fermentability on *t*10, *c*12 CLA also follow the pattern of pH changes. After 12 h of incubation, the *t*10, *c*12 CLA concentration in high starch cultures containing HMC was 71% higher at low pH (0.42 vs. 0.24 g/100 g), and 35% lower at high pH (0.12 vs. 0.19 g/100 g) than in high starch cultures containing DC. However, the pH differences between high starch cultures containing HMC and DC were only -0.02 and -0.03 at low pH and high pH, respectively. These results show that the effect of starch fermentability on BH might not only be mediated through pH changes. We previously discussed that HMC might have

increased culture pH by increasing microbial N (Herrera-Saldana et al., 1990), or that specific bacterial species primarily utilizing fermentable carbohydrate depended on the culture pH and starch content (Mackie et al., 1979). *Megasphaera elsdenii*, a *t*10, *c*12 CLA producer (Kim et al., 2002), might be more abundant in high starch cultures containing HMC and led to high *t*10, *c*12 CLA at 12 h of incubation. Among the cellulolytic bacteria, *Butyrivibrio fibrisolvens* and *Bacteroides succinogenes* have higher tolerance of low pH than *Ruminococcus albus*, *Ruminococcus flavefaciens* (Russell and Dombrowski, 1980). At low culture pH, high starch cultures containing HMC might have increased mass of specific bacteria, such as *Butyrivibrio fibrisolvens* and *Megasphaera elsdenii*, which biohydrogenate LA, but decreased mass of those more pH sensitive cellulolytic bacteria due to the low culture pH. This could explain why HMC decreased both OBCFA and NDF disappearance extent, but increased BH extent, compared with high starch cultures containing DC. In low pH cultures, HMC decreased *t*10, *c*12 CLA and increased *t*10 18:1, but did not affect the concentration of 18:0. Previous research has shown that low pH is associated with decreased formation of 18:0 (Sun et al., 2014, Fuentes et al., 2009, Troegeler-Meynarier et al., 2006), which might be caused by inhibition of low pH on the 18:0-producer, *Butyrivibrio proteoclasticus* (Wallace et al., 2006).

However, at high pH, high starch cultures containing HMC had a lower appearance rate of *t*10, *c*12 CLA than those at low pH, which resulted in the lowest *t*10, *c*12 CLA and highest *t*10 18:1 concentrations of all cultures after 24 h of incubation. The changes in *t*10, *c*12 CLA concentration follow the pattern of pH fluctuation of these cultures. Russell and Dombrowski (1980) found that cell yield of *Megasphaera elsdenii* increased with decreasing pH starting at pH 6.7 and peaked around pH 5.7, which explains the lower *t*10, *c*12 CLA content in high pH, compared to low pH, cultures. At high pH, high starch content, combined with HMC, increased

the pH of cultures, which might have increased the presence of *Butyrivibrio* spp., the major bacteria that hydrogenates *t*10, *c*12 CLA and *c*9, *t*11 CLA to *t*10 18:1 and *t*11 18:1 (McKain et al., 2010). Our *t*10 18:1 concentration results are consistent with this assumption. Lascano et al. (2016) tested the effect of a treatment containing 21% starch with different degradabilities on BH at pH over 6.0 and found that highly degradable starch increased *t*10, *c*12 CLA 8 h after feeding. This is consistent with our results including that, compared with DC, low starch cultures with HMC increased *t*10, *c*12 CLA at high pH after 12 h of incubation.

CONCLUSIONS

We utilized an in vitro batch culture system to test the interactive effects of starch fermentability, starch content, and culture pH on FA biohydrogenation. Similar to our previous work, culture pH proved to be the major factor, which influenced NDF disappearance extent and BH, with low pH decreasing NDF disappearance extent and BH extent, and increasing formation of *t*10, *c*12 CLA. High starch content influenced NDF disappearance extent and BH by decreasing pH of the cultures. Starch fermentability interacted with starch content and culture pH to affect BH of FA. Specifically, high starch cultures containing HMC increased *t*10, *c*12 CLA concentration greater at low pH than at high pH. Therefore, the effect of HMC was dependent on the culture pH.

APPENDIX

APPENDIX

Table 3.1. Ingredient and FA compositions of substrates used in in vitro batch cultures[1]

	Substrates			
	Low starch		High starch	
	DC	HMC	DC	HMC
Ingredient, % DM				
Alfalfa hay[2]	70	70	55	55
DC[3]	30	-	45	-
HMC[4]	-	30	-	45
Total FA, % DM	3.43	3.5	3.45	3.49
FA, g/100 g				
16:0	15.8	16.1	15.3	15.6
18:0	2.48	2.42	2.33	2.33
*c*9 18:1	22.4	23.2	22.8	23.9
LA[5]	47.9	47.2	50.1	48.7
*c*9, *c*12, *c*15 18:3	4.98	4.95	4.19	4.15
∑Others	6.35	6.14	5.16	5.3

[1] Average of two replicates.
[2] Alfalfa hay was treated with corn oil (2% DM) to increase total UFA content in substrate.
[3] DC = dry ground corn.
[4] HMC = high moisture corn.
[5] LA, *c*9, *c*12 18:2.

Table 3.2. pH, NDF disappearance extent, total FA content, and BH extent of LA of in vitro batch cultures after 24 h of incubation[1].

Item	Low Starch[2]				High Starch				SEM	*P*-values[3]
	DC		HMC		DC		HMC			
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH		
pH	5.83	6.03	5.87	6.05	5.70	5.91	5.74	6.00	0.01	CpH**, SC**, SF**, SC × CpH *
NDF disappearance extent, %	13.1	30.7	11.4	31.2	11.1	30.0	8.50	22.7	0.96	CpH **, SC**, SF**, SC × SF *, SC × SF × CpH *
Total FA, mg/culture	22.5	22.6	23.0	22.3	23.1	22.4	23.3	21.8	0.19	CpH**, SC × CpH **, SF × CpH *
BH extent of LA[4], %	27.9	69.2	33.0	62.8	26.1	60.9	30.6	53.1	0.84	CpH**, SC**, SC × CpH **, SF × CpH **

[1] Values are means of 2 replicates for all variables, except pH value is the mean of 4 replicates.
[2] Low starch, 22% DM of starch content in substrates; high starch, 33% DM of starch content in substrates; DC, dry ground corn; HMC, high moisture corn; low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2.
[3] CpH, effect of culture pH (low pH & high pH); SC, effect of starch content level (low starch, 22% DM of starch; high starch, 33% DM of starch); SF, effect of starch fermentability (DC, dry ground corn; HMC, high moisture corn); SC × CpH, interaction of starch content and culture pH; SF × CpH, interaction of starch fermentability and culture pH; SF × SC, interaction of starch fermentability and starch content; SC × SF × CpH, interaction of starch content, starch fermentability and culture pH; *, $P < 0.05$; **, $P < 0.01$.
[4] BH extent of LA, was calculated by subtracting the amount of LA in the cultures at 24 h from the amount at 0 h and dividing by the amount at 0 h.

Table 3.3. Increasing rates of NDF disappearance extent and BH extent of LA from 12 to 24 h of incubation[1].

Increasing rate, %/h	Low Starch				High Starch			
	DC		HMC		DC		HMC	
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH
NDF disappearance extent	0.57	1.03	0.51	1.43	0.53	1.24	0.50	1.09
BH extent of LA[3]	1.15	2.00	1.34	1.73	1.08	1.95	0.97	1.33

[1] Increasing rates of NDF disappearance extent and BH extent from 12 to 24 h of incubation were calculated by dividing difference between NDF disappearance extent or BH extent at 12 and 24 h by 12 h.
[2] Low starch, 22% DM of starch content in substrates; high starch, 33% DM of starch content in substrates; DC, dry ground corn; HMC, high moisture corn; low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2.
[3] BH extent of LA, was calculated by subtracting the amount of LA in the cultures at 24 h from the amount at 0 h and dividing by the amount at 0 h.

Table 3.4. Concentrations of selected FA in in vitro batch cultures after 24 h of incubation[1].

FA, g/100 g total FA	High Starch[2]				Low Starch				SEM	*P*-values[3]
	DC		HMC		DC		HMC			
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH		
12:0	0.66	0.53	0.67	0.53	0.74	0.60	0.70	0.57	0.01	CpH **, SC **, SC × SF *
iso 13:0	0.15	0.15	0.15	0.16	0.13	0.14	0.12	0.13	0.003	CpH*, SC**, SC × SF *
13:0	0.09	0.12	0.08	0.10	0.10	0.11	0.09	0.08	0.004	CpH **, SF**, SC × CpH **
14:0	1.14	1.40	1.16	1.31	1.15	1.44	1.11	1.26	0.02	CpH**, SF**, SC × SF*, SF × CpH **
iso 15:0	0.29	0.35	0.28	0.34	0.30	0.36	0.27	0.33	0.01	CpH**, SF**
anteiso 15:0	1.29	1.88	1.26	1.88	1.38	1.93	1.20	1.87	0.03	CpH**, SF**
15:0	0.69	1.06	0.67	0.95	0.71	0.98	0.64	0.83	0.02	CpH**, SC**, SF**, SC × CpH **, SF × CpH**
iso 16:0	0.13	0.16	0.12	0.15	0.13	0.15	0.13	0.13	0.003	CpH**, SC*, SF**, SC × CpH **, SF × CpH *
16:0	16.7	16.7	16.6	16.8	16.2	16.3	16.3	16.3	0.05	SC**
*c*7+*c*8 16:1	0.26	0.14	0.29	0.17	0.22	0.13	0.18	0.10	0.01	CpH**, SC**, SC × SF **, SC × CpH *
*c*9 16:1	0.22	0.18	0.22	0.20	0.24	0.23	0.22	0.18	0.004	CpH**, SC*, SF**, SC × SF**, SC × SF × CpH **
*c*10+*t*13 16:1	0.16	0.19	0.16	0.17	0.16	0.20	0.14	0.17	0.004	CpH**, SF**, SC × SF*, SF × CpH*
18:0	13.1	23.4	12.9	19.0	13.0	20.1	13.1	16.1	0.31	CpH**, SC**, SF**, SC × CpH**, SF × CpH**
*t*4 18:1	0.02	0.07	0.02	0.05	0.02	0.04	0.01	0.04	0.01	CpH**, SC**
*t*5 18:1	0.02	0.07	0.02	0.05	0.02	0.05	0.02	0.05	0.002	CpH**, SC**, SF**, SC ×

Table 3.4. (cont'd)

FA, g/100 g total FA	High Starch[2]				Low Starch				SEM	*P*-values[3]
	DC		HMC		DC		HMC			
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH		
*t*6 + *t*7 + *t*8 18:1	0.21	0.70	0.16	0.64	0.20	0.60	0.13	0.54	0.01	SF*, SC × CpH**, SF × CpH**, SC × SF × CpH* CpH**, SC**, SF**, SC × CpH**
*t*9 18:1	0.39	0.70	0.38	0.70	0.37	0.62	0.36	0.64	0.01	CpH**, SC**, SC × CpH**, SF × CpH*
***t*10, 18:1**	2.30	1.60	3.48	1.77	2.70	1.85	3.93	2.37	0.08	CpH**, SC**, SF**, SF × CpH*
***t*11, 18:1**	6.67	13.1	7.59	13.4	5.65	11.4	5.86	12.3	0.22	CpH**, SC**, SF**
*t*12 18:1	0.43	1.04	0.43	1.02	0.42	0.90	0.41	0.90	0.01	CpH**, SC**, SC × CpH*
***c*9, 18:1**	17.6	13.0	17.7	14.7	17.2	14.5	17.7	16.2	0.16	CpH**, SC**, SF**, SC × CpH**, SF × CpH**
*c*11 18:1	1.32	1.84	1.25	1.93	1.27	2.02	1.24	2.06	0.02	CpH**, SC**, SC × CpH**, SF × CpH**
*c*12 18:1	0.48	0.91	0.64	1.00	0.59	0.91	0.88	0.99	0.02	CpH**, SC**, SF**, SC × CpH**, SF × CpH**
*c*13 18:1	0.04	0.05	0.04	0.05	0.03	0.04	0.03	0.04	0.002	CpH**, SC**
*c*14 + *t*16 18:1	0.14	0.34	0.13	0.26	0.14	0.28	0.135	0.21	0.01	CpH**, SC**, SF**, SC × CpH**, SF × CpH**
*c*15 18:1 + 19:0										
*t*8, *t*10 + *t*9, *t*11 + *t*10, *t*12 18:2	0.41	0.57	0.15	0.50	0.32	0.57	0.23	0.33	0.06	CpH**, SF**
*t*9, *t*12 18:2	0.17	0.12	0.20	0.15	0.15	0.13	0.17	0.15	0.005	CpH**, SC*, SF**, SC × CpH**

Table 3.4. (cont'd)

FA, g/100 g total FA	High Starch[2]				Low Starch				SEM	*P*-values[3]
	DC		HMC		DC		HMC			
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH		
*c*9, *t*12 18:2	0.44	0.30	0.47	0.37	0.40	0.32	0.44	0.40	0.01	CpH**, SF**, SC × CpH**, SF × CpH**
*t*9, *c*12 18:2	0.39	0.29	0.42	0.36	0.33	0.29	0.40	0.37	0.01	CpH**, SF**, SC × CpH*
***c*9, *t*11 CLA**	0.20	0.56	0.16	0.36	0.18	0.58	0.13	0.30	0.02	CpH**, SF**, SF × CpH**
LA[4]	26.0	11.3	24.3	13.7	27.4	15.2	25.6	17.7	0.40	CpH**, SC**, SC × CpH**, SF × CpH**
*c*10, *c*12 CLA	0.04	0.04	0.04	0.04	0.03	0.04	0.03	0.04	0.003	CpH*, SC*
***t*10, *c*12 CLA**	0.31	0.22	0.21	0.22	0.32	0.27	0.33	0.18	0.01	CpH**, SC**, SF**, SC × CpH*, SC × SF × CpH**
*c*11, *c*13 CLA	0.09	0.13	0.11	0.11	0.11	0.11	0.11	0.07	0.01	SC × SF*, SC × CpH**, SF × CpH**
*t*11, *t*13 CLA	0.07	0.06	0.06	0.07	0.06	0.06	0.05	0.04	0.004	SC**, SF*, SC × SF × CpH*
***c*9, *c*12, *c*15 18:3**	1.82	1.07	1.83	1.21	1.64	1.04	1.65	1.18	0.02	CpH**, SC**, SF**, SC × CpH**, SF × CpH**
19:0	0.21	0.23	0.22	0.22	0.19	0.22	0.18	0.21	0.01	CpH**, SC**
20:0	0.53	0.55	0.52	0.54	0.48	0.51	0.49	0.50	0.01	CpH**, SC**
*c*11 20:1	0.19	0.16	0.20	0.17	0.18	0.17	0.20	0.18	0.004	CpH**, SC*, SF**, SC × CpH**
*c*11, *c*14 20:2	0.06	0.05	0.06	0.05	0.06	0.05	0.06	0.05	0.003	CpH**
*c*5, *c*8, *c*11 20:3	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.003	SC*
22:0	0.37	0.39	0.36	0.39	0.30	0.33	0.31	0.33	0.01	CpH*, SC**

Table 3.4. (cont'd)

FA, g/100 g total FA	High Starch[2]				Low Starch				SEM	*P*-values[3]
	DC		HMC		DC		HMC			
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH		
23:0	0.06	0.07	0.06	0.07	0.06	0.06	0.07	0.08	0.005	CpH*, SC × SF*
24:0	0.30	0.33	0.28	0.33	0.24	0.29	0.28	0.31	0.02	
Unknown	3.93	3.88	4.20	3.81	4.38	4.07	4.41	3.46	0.14	CpH**, SF × CpH*
Total *trans* 18:1	10.0	17.3	11.9	17.8	9.5	15.4	10.8	16.7	0.32	CpH**, SC**, SF**
∑ CLA [5]	1.10	1.58	0.71	1.30	1.01	1.62	0.89	0.93	0.06	CpH**, SF**, SC × CpH*, SF × CpH*, SC × SF × CpH**
BCFA	1.85	2.57	1.83	2.51	1.92	2.60	1.72	2.44	0.04	CpH**, SF**
OBCFA[6]	2.88	3.92	2.79	3.78	2.91	3.90	2.57	3.62	0.05	CpH**, SC*, SF**, SC × SF*

[1] Values are means of 2 replicates for all variables.
[2] Low starch, 22% DM of starch content in substrates; high starch, 33% DM of starch content in substrates; DC, dry ground corn; HMC, high moisture corn; low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2.
[3] CpH, effect of culture pH (low pH & high pH); SC, effect of starch content level (low starch, 22% DM of starch; high starch, 33% DM of starch); SF, effect of starch fermentability (DC, dry ground corn; HMC, high moisture corn); SC × CpH, interaction of starch content and culture pH; SF × CpH, interaction of starch fermentability and culture pH; SF × SC, interaction of starch fermentability and starch content; SC × SF × CpH, interaction of starch content, starch fermentability and culture pH; *, $P < 0.05$; **, $P < 0.01$.
[4] LA, linoleic acid (*c*9, *c*12 18:2).
[5] CLA, conjugated linoleic acids.
[6] OBCFA , odd and branched chain fatty acid, including 13:0, *iso* 13:0, *iso* 14:0, 15:0, *iso* 15:0, *anteiso* 15:0, *iso* 16:0, *anteiso* 17:0.

Table 3.5. Appearance rates of *t*10, *c*12 CLA and *t*10 18:1 from 0 to 12 h and from 12 to 24 h of incubation[1].

Increasing rate, g/100 g/h	Time	Low Starch[2]				High Starch			
		DC		HMC		DC		HMC	
		Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH
*t*10, *c*12 CLA	0 to 12 h	0.016	0.005	0.022	0.007	0.017	0.013	0.030	0.007
	12 to 24 h	0.007	0.011	-0.010	0.008	0.006	0.007	-0.007	0.004
*t*10 18:1	0 to 12 h	0.065	0.055	0.071	0.061	0.073	0.062	0.106	0.074
	12 to 24 h	0.104	0.060	0.196	0.067	0.133	0.068	0.204	0.101

[1] Appearance rates of *t*10, *c*12 CLA and *t*10 18:1 were calculated as: FA concentration increase over each 12 h incubation period (0 to 12 h or 12 to 24 h) divided by 12 h.
[2] Low starch, 22% DM of starch content in substrates; high starch, 33% DM of starch content in substrates; DC, dry ground corn; HMC, high moisture corn; low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2

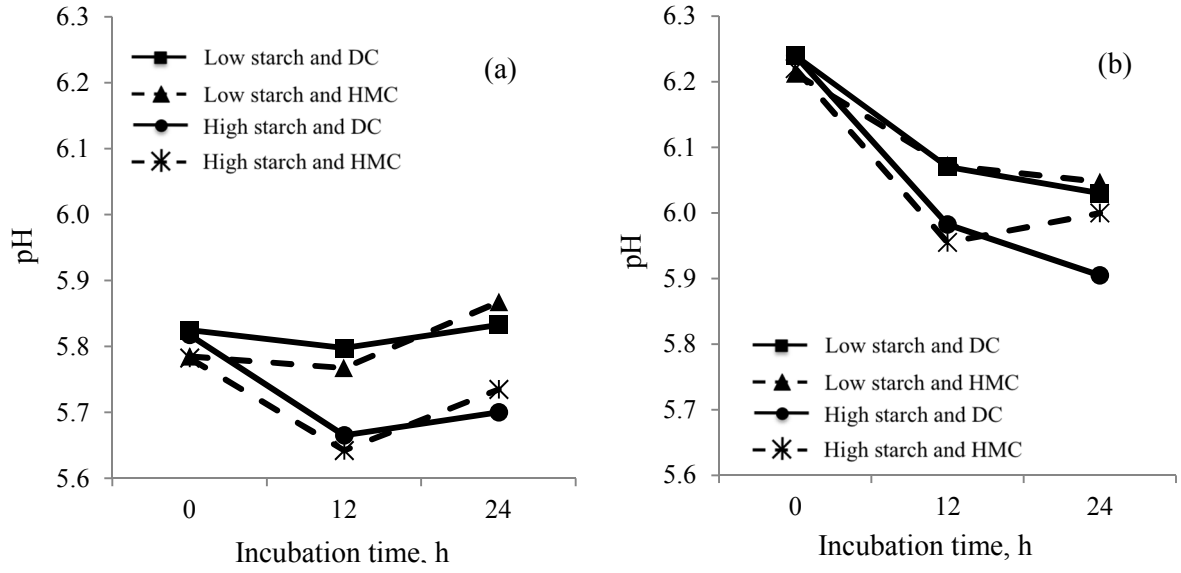

Figure 3.1.pH of cultures incubated with low starch and DC, low starch and HMC, high starch and DC, high starch and HMC at low pH (a) and high pH (b) during 24 h of incubation.

pH of cultures incubated with low starch and DC ( ), low starch and HMC ( ), high starch and DC ( ), high starch and HMC ( ) at low pH (a) and high pH (b) during 24 h of incubation (SEM = 0.01; $P = 0.01$ for interaction effect of starch content, starch fermentability, culture pH and time).

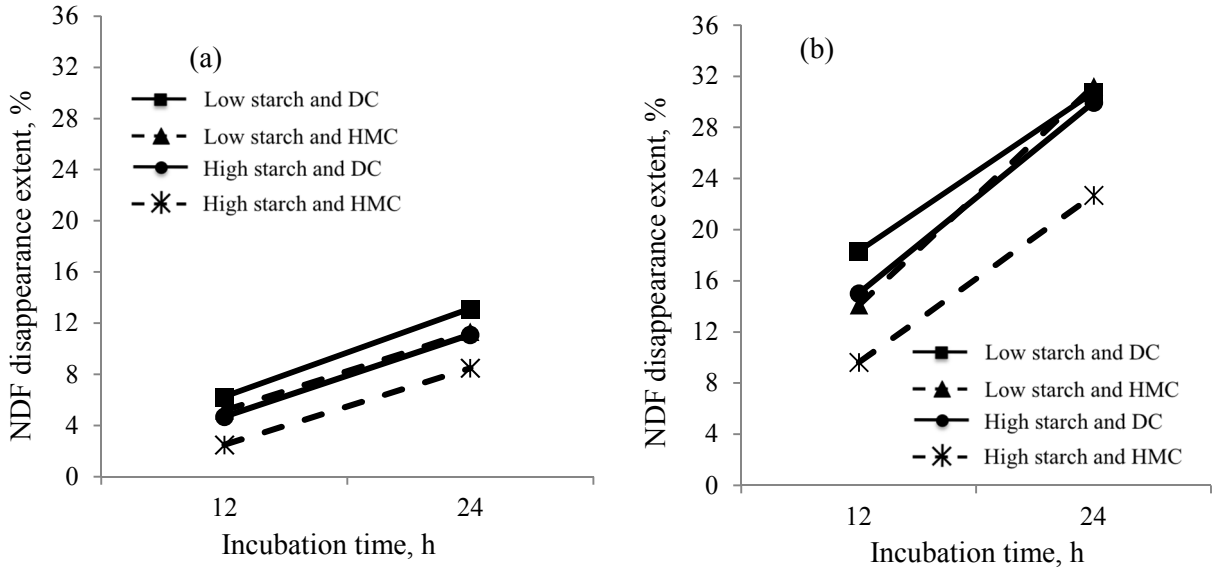

Figure 3.2. NDF disappearance extent in cultures incubated with low starch and DC, low starch and HMC, high starch and DC, high starch and HMC at low pH (a) and high pH (b) during 24 h of incubation.

NDF disappearance extent in cultures incubated with low starch and DC ( ), low starch and HMC ( ), high starch and DC ( ), high starch and HMC ( ) at low pH (a) and high pH (b) during 24 h of incubation (SEM = 0.01; $P = 0.08$ for interaction effect of starch content, starch fermentability, culture pH and time). The NDF disappearance extent was calculated by subtracting the amount of NDF residue in the cultures at 12 and 24 h from the amount at 0 h and dividing by the amount at 0 h.

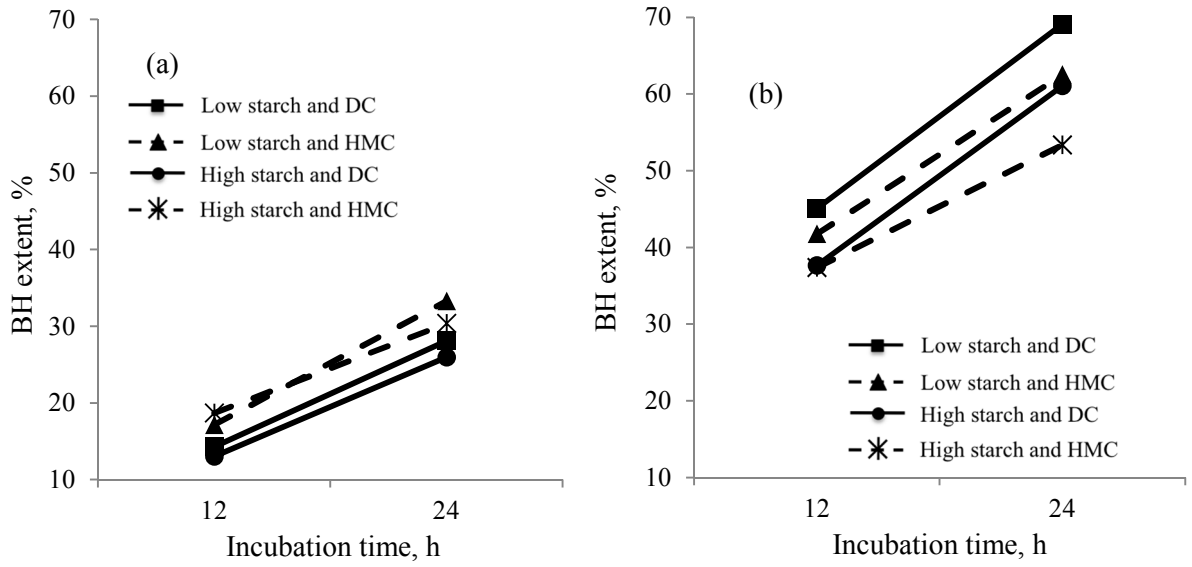

Figure 3.3. BH extent of LA in cultures incubated with low starch and DC, low starch and HMC, high starch and DC, high starch and HMC at low pH (a) and high pH (b) during 24 h of incubation.

BH extent of LA (*c*9, *c*12 18:2) in cultures incubated with low starch and DC ( ), low starch and HMC ( ), high starch and DC ( ), high starch and HMC ( ) at low pH (a) and high pH (b) during 24 h of incubation (SEM = 0.01; $P < 0.001$ for interaction effect of starch content, starch fermentability, culture pH and time). The BH extent was calculated by subtracting the amount of LA in the cultures at 12 and 24 h from the amount at 0 h and dividing by the amount at 0 h.

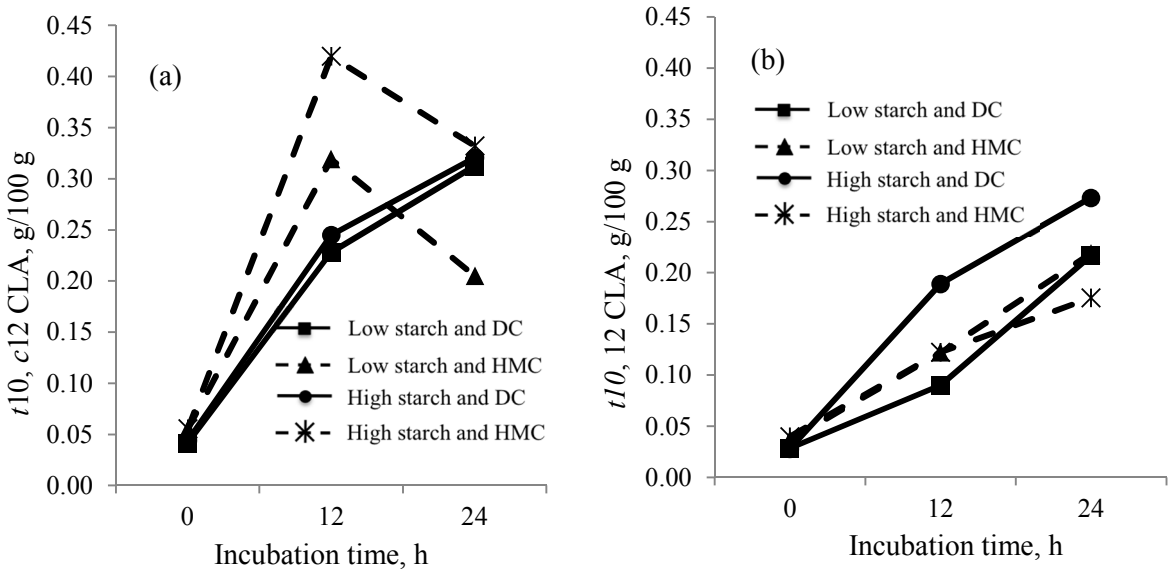

Figure 3.4. Concentration of *t*10, *c*12 CLA in cultures incubated with low starch and DC, low starch and HMC, high starch and DC, high starch and HMC at low pH (a) and high pH (b) during 24 h of incubation.

Concentration of *t*10, *c*12 CLA in cultures incubated with low starch and DC ( ), low starch and HMC ( ), high starch and DC ( ), high starch and HMC ( ) at low pH (a) and high pH (b) during 24 h of incubation (SEM = 0.01; $P < 0.001$ for interaction effect of starch content, starch fermentability, culture pH and time).

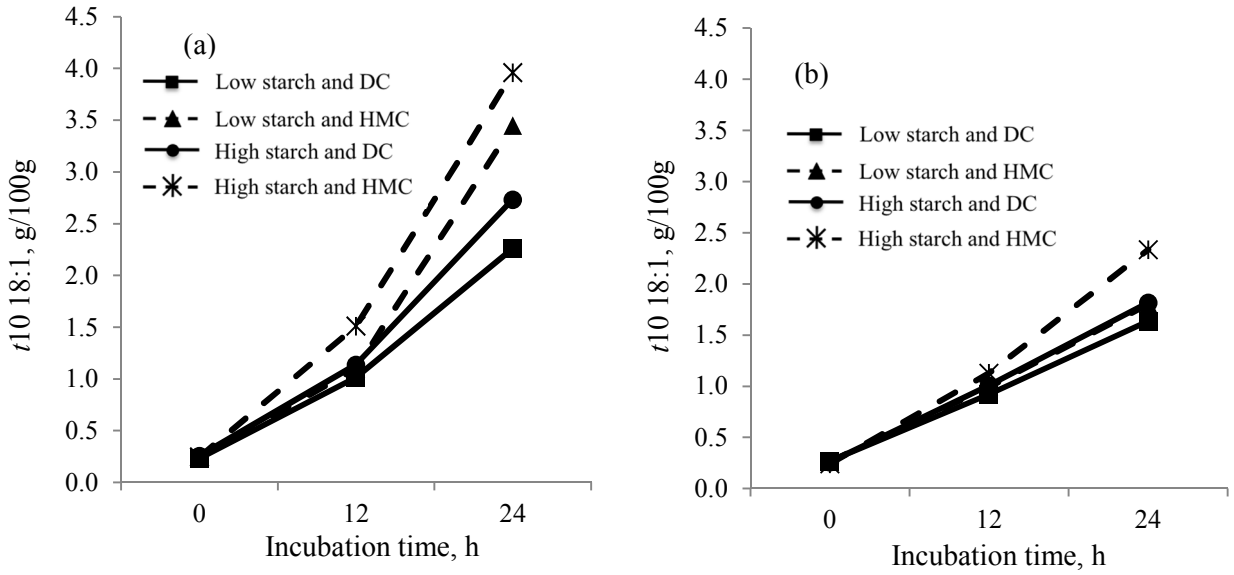

Figure 3.5. Concentration of *t*10 18:1 in cultures incubated with low starch and DC, low starch and HMC, high starch and DC, high starch and HMC at low pH (a) and high pH (b) during 24 h of incubation.

Concentration of *t*10 18:1 in cultures incubated with low starch and DC ( ), low starch and HMC ( ), high starch and DC ( ), high starch and HMC ( ) at low pH (a) and high pH (b) during 24 h of incubation (SEM = 0.01; $P = 0.10$ for interaction effect of starch content, starch fermentability, culture pH and time).

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CHAPTER 4

EFFECTS OF RUMEN INOCULUM ADAPTED AND UNADAPTED TO *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCTS, CULTURE PH, AND STARCH FERMENTABILITY ON THE BIOHYDROGENATION OF UNSATURATED FATTY ACIDS IN BATCH CULTURE

INTRODUCTION

Milk fat is a major contributor to milk price and diet-induced milk fat depression (MFD) often results in significant reduction of farm income. Current evidence indicates that MFD is caused by changes in rumen biohydrogenation (BH) that result in the production of specific intermediates (*e.g.* *t*10, *c*12 18:2 conjugated linoleic acid, CLA) which reduce milk fat synthesis in the mammary gland by altering gene expression (Bauman et al., 2011). Dietary and ruminal factors can have variable influences on the rumen BH pathways that increase production of MFD-related intermediates, and thus, the risk of MFD.

Rumen pH is highly correlated with milk fat percentage (Allen, 1997). Previous studies have reported that reducing rumen pH can alter BH pathways and increase formation of BH intermediates associated with MFD (Troegeler-Meynadier et al., 2003; Sun et al., 2014, 2015). Diets high in fermentable starch (*e.g.* high moisture corn) increase the risk of decreased milk fat yield (Oba and Allen, 2003; Bradford and Allen, 2004) and have been used experimentally to induce MFD (Longuski et al., 2009). We recently reported that in vitro cultures containing 33% starch from high moisture corn increased formation of *t*10, *c*12 CLA at low culture pH, and that culture pH had a greater effect on BH of *c*9, *c*12 18:2 (LA) than did starch fermentability (Sun et al., 2015).

A recent meta-analysis showed that *Saccharomyces cerevisiae* fermentation product (SCFP), manufactured by Diamond V and supplemented as a dietary feed additive, increased milk fat yield (Poppy et al., 2012). Longuski et al. (2009) reported that SCFP prevented MFD

when diet fermentability was altered over a short period of time. Potential anti-MFD mechanisms of SCFP may include the effect of the supplement on the metabolism of rumen microbes and stabilization of ruminal fermentation (Harrison et al., 1988; Miller-Webster et al., 2002). Both mechanisms may help minimize the risk of MFD; however, there is limited information on the effect of SCFP on BH pathways and formation of BH intermediates associated with MFD.

In vitro batch culture and continuous culture incubations are commonly used in rumen fermentation and microbial metabolism research due to advantages of low cost and flexible, well-controlled conditions (Boguhn et al., 2014; Zened et al., 2011; Vlaeminck et al., 2008). However, the most common incubation time (≤ 24 h) is markedly less than the time required for ruminal microbes to reach equilibrium following a dietary change (Weimer et al., 2010). In respect of this limitation, a small number of studies have successfully used rumen inoculum from cows fed different diets as an additional treatment variable in order to examine the relationship among dietary ingredients and rumen conditions on fermentation and BH (Vlaeminck et al. 2008; Zened et al., 2011). Our group successfully developed an in vitro batch culture system (Sun et al., 2014, 2015), with varied pH and FA analysis completed in the same culture tube, to allow more accurate and precise FA analysis results. In our current study, to ensure that rumen microbes had time to adapt to SCFP, we supplemented SCFP to donor cows and utilized their rumen fluid as inoculum. Our objective, therefore, was to examine the effects of rumen fluid inoculum, either unadapted or adapted to SCFP, on the BH of unsaturated FA at two culture pH levels and two starch sources with different fermentabilities. We hypothesized that rumen inoculum adapted to SCFP would increase the extent of BH of unsaturated FA and decrease production of MFD-associated BH intermediates.

MATERIALS AND METHODS

Cow Feeding

Six rumen-fistulated lactating Holstein dairy cows (DIM 228 ± 17) were used in a crossover design with two 28-d cow treatment periods and a 14-d washout period in between. During period 1, half of the donor cows (n=3) were fed a control diet and the other half (n=3) were fed the same diet top dressed with SCFP (14 g/d of Diamond V XPC^{TM}, Diamond V, Cedar Rapids, IA). The diet contained approximately 50:50 forage:concentrate, and was formulated according to NRC (2001) recommendations. The ingredient and nutrient composition of the diet fed as a TMR is described in Table 4.1. All cows were fed the control diet without SCFP supplementation during the washout period, and switched between control and SCFP-supplemented diets during period 2. In vitro batch culture incubations were performed at the end of both cow treatment periods (d 28).

In vitro batch cultures

Rumen fluid and digesta were manually collected from the ventral rumen one hour after feeding on d 28 of each cow treatment period. Rumen fluid and digesta from cows fed the same diet were mixed in equal proportions and transferred into pre-warmed Thermos containers. After collection, rumen fluid and digesta were blended for 15 s in a 1-gallon Waring blender, to detach bacteria from feed particles, and passed through a Buchner funnel lined with nylon mesh and glass wool to filter out feed particles. Strained rumen fluid was used as inoculum to run culture incubations. Batch cultures were run in a randomized design with a $2 \times 2 \times 2$ factorial arrangement of treatments: two in vitro pH levels (low pH = 5.8 or high pH = 6.2), two types of rumen fluid (SCFP adapted [A-RF] or unadapted [U-RF]), and two starch sources (dry ground corn [DC] or high moisture corn [HMC]). All cultures were run in quadruplicate. Culture

substrate contained (DM basis) 55% alfalfa hay as a forage source and 45% DC or HMC as starch sources to provide 33% starch (Table 4.2). To increase total unsaturated FA content of substrates, corn oil was added at 2% DM to the alfalfa hay by dissolving it in ethanol and spraying it onto dried and ground alfalfa hay. Alfalfa hay and corn grain sources were dried at 55°C with a forced-air oven. Dry ground corn and high moisture corn were ground through the 1-mm screen of an abrasion mill (UDY Corp., Fort Collins, Colorado) and alfalfa hay was ground through the 1-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Total FA content and FA profile of substrates are shown in Table 4.2.

All cultures were prepared as described by Goering and Van Soest (1970). Cultures were maintained in 100-mL Pyrex centrifuge tubes containing 500 mg of substrate (Table 2), 40 mL of buffer medium, 2 mL of reducing solution, and 10 mL of strained rumen inoculum collected from U-RF or A-RF cows. The weight of substrates was recorded for each culture tube. Sayre and Van Soest (1972) previously reported that large centrifuge tubes yield similar NDF digestibility results compared to the more commonly used 125-mL Erlenmeyer flask. Culture tubes were flushed with CO_2 and sealed with 5.5-cm rubber stoppers connected to a Bunsen valve by a glass tube. All cultures were maintained in a 39°C water bath. Buffer medium of pH 5.8 and 6.2 was achieved by adjustment with 1 M citric acid as described by Grant and Mertens (1992).

Cultures tubes were collected at 0, 12, and 24 h of incubation for both periods. At sampling time, pH was measured on all four replicates/period with a pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland). Once pH was determined, incubations were terminated by placing culture tubes in an ice bath and adding dry ice to the tubes. Culture tubes for NDF residue analysis (2 replicates/period) were placed in a 4°C cooler until analysis (completed

within 48 h). Culture tubes for FA composition analysis (2 replicates/period) were stored at -20°C and subsequently freeze-dried directly in the culture tubes.

Sample Analysis

NDF residue was analyzed as described by Merten (2002). To ensure the accuracy of FA composition analysis, samples were freeze-dried and methylated directly in culture tubes using a 2-step methylation protocol adapted from Jenkins (2010). 17:0 (1:1 mg/mL toluene) was added to cultures as internal standard after incubations were terminated and prior to storing at -20°C. All tubes were pre-weighed and re-weighed after being freeze-dried to determine culture content weight. Freeze dried samples were mixed with 8 mL of 0.5 M sodium methoxide solution in methanol and incubated for 10 min in a 50°C water bath. After tubes had cooled, 12 mL of 5% methanolic HCl solution was added before 10 min of incubation in a 80°C water bath. After tubes were removed from the water bath and allowed to cool, 10 mL n-hexane and 30 mL 6% K_2CO_3 solution were added and the solutions were mixed by vortexing. Next, tubes were centrifuged for 10 min at 1620 × g and the hexane layer containing FAME was transferred to a 15-mL centrifuge tube containing 2 g sodium sulfate. FAME were extracted again by adding 5 mL hexane to the culture tubes and repeating the mixing, centrifuging, and transferring steps above. The FAME solution was subsequently filtered through silica gel and charcoal to remove any remaining sodium sulfate and the hexane was removed with nitrogen flow at 37°C. The FAME samples were weighed and a 1% solution was prepared with n-hexane based on weight. The 1% FAME-solution was transferred to 2-mL GLC vials for analysis.

FA composition was determined by a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with a split injector (1:100 split ratio) and a flame-ionization detector (FID) using a CP-Sill 88 WCOT (wall-coated open tubular) fused-silica column (100 m × 0.25 mm i.d. × 0.2-

μm film thickness; Varian Inc., Lake Forest, CA). The carrier gas was hydrogen at a flow rate of 1 mL/min. Hydrogen, purified air, and nitrogen makeup gas were used as the FID gases at flow rates of 40, 400, and 30 mL/min, respectively. Injector and detector temperatures were 270°C. The oven program was: initial temperature held for 0.5 min at 40°C, programmed to increase to 155°C at 25°C/min and held for 30 min, and then increased to 215°C at 4°C/min and held for 35 min. Injection volume was 1 μL. FID response was the basis for integration and quantification (GCsolution software version 2.32.00; Shimadzu). Known FAME standards (GLC reference standard 463, GLC reference standard 481-B, and conjugated octadecadienoic mixture #UC-59-M from Nu-Chek Prep Inc., Elysian, MN; Supelco 37 component FAME mix, *cis*/*trans* FAME mix, bacterial acid methyl ester mix, and PUFA No. 2 mix from Supelco Inc., Bellefonte, PA) were used for determination of individual FAME by comparing retention times. Quantification of FA composition covered approximately 45 FA in the range of C12:0 to C24:0.

Calculations and Statistical Analysis

NDF disappearance extent was calculated by subtracting the content of NDF in the cultures at 12 and 24 h from the content at 0 h, and dividing by the content at 0 h. The extent of BH for LA was calculated by subtracting the content of LA in the cultures at 12 and 24 h from the content at 0 h, and dividing by the amount at 0 h. Appearance rates of individual FA were calculated for *t*10, *c*12 CLA and *t*10 18:1 as follows: FA concentration increase in each 12-h incubation period (0 to 12 h or 12 to 24 h) divided by 12 h.

All data from in vitro batch cultures were analyzed by using the fit model procedure of JMP (Version 10, SAS Institute). The pH, NDF disappearance extent, extent of BH, total FA content, and individual FA concentrations of in vitro batch cultures at 24 h of incubation were analyzed using a model including main effects of rumen fluid-type, in vitro culture pH level,

starch source, period, and the interactions among main effects. Additionally, to test the effect of treatment across time on NDF disappearance extent, extent of BH, *t*10, *c*12 CLA concentration, and *t*10 18:1 concentration, results obtained at each sampling time (0, 12, and 24 h of incubation) were analyzed separately using the same model but with the added effect of time and its interactions with the other main effects included. Interactions were removed from the model if $P > 0.15$. Least square means with standard error are reported, and significance declared at $P < 0.05$.

RESULTS

FA concentrations of substrates and rumen inoculum from donor cows

Total FA content and FA concentrations were consistent between substrates containing dry ground corn or high moisture corn (Table 4.2). Rumen fluid pH and FA concentrations collected from cows fed control (U-RF) and SCFP-supplemented diets (A-RF) are shown in Table 4.3. Compared to the control diet alone, the SCFP supplement decreased rumen fluid pH and increased total FA content and concentrations of LA and *t*10, *c*12 CLA, and decreased concentrations of 18:0, *t*11 18:1 and *c*11 18:1 in rumen fluid.

pH, NDF disappearance extent, and BH extent of in vitro cultures

The effect of treatment on pH, NDF disappearance extent, and BH extent of LA in culture after 24 h of incubation is shown in Table 4. Changes in these variables during the 24-h incubation period are shown in Figures 4.1-4.3. Overall, the pH of all cultures decreased during incubation. However, the difference between low pH and high pH treatments remained greater than 0.2 units across time, and the mean values for low and high pH cultures throughout the 24-h incubation were 5.7 and 6.0 (SEM = 0.003), respectively ($P < 0.001$, Figure 4.1). DC and A-RF

decreased pH compared with HMC (5.75 vs. 5.79, $P < 0.001$) and U-RF (5.75 vs. 5.79, $P < 0.001$), respectively.

Overall, compared with high pH, low pH decreased NDF disappearance extent (10.1 vs. 16.2%, $P < 0.001$) and BH extent of LA (32.3 vs. 47.3%, $P < 0.001$) after 24 h of incubation (Table 4.4). A-RF increased NDF disappearance extent (13.9 vs. 12.4%, $P < 0.001$) and BH extent (41.3 vs. 38.3%, $P < 0.001$), compared with U-RF. DC increased NDF disappearance extent (14.4 vs. 11.9%, $P < 0.001$) compared with HMC. Starch source had no main effect on BH extent but interacted with culture pH ($P = 0.008$), with a lower BH extent for DC at low pH compared with HMC at low pH (30.7 vs. 34.0%, $P < 0.01$). However, there was no difference between starch sources at high pH (47.6 vs. 46.9%). We observed interactions between culture pH and starch source, and culture pH and rumen fluid, on NDF disappearance extent (all $P < 0.05$). Compared with HMC, DC increased NDF disappearance extent (10.7 vs. 9.5%, $P < 0.05$) at low pH, but the magnitude of increase was greater (18.1 vs. 14.3%, $P < 0.01$) at high pH. A-RF increased NDF disappearance extent at high pH (17.4 vs. 15.0%, $P < 0.01$) compared with U-RF, but there was no difference between A-RF and U-RF at low pH (10.5 vs. 9.7%). High pH decreased total FA content of cultures by 1.8% compared with low pH ($P < 0.001$).

Changes in NDF disappearance extent and BH extent of LA during 24 h of incubation are shown in Figures 4.2 and 4.3, respectively. Compared with low pH, high pH increased both NDF disappearance extent (0.70 vs. 0.38%/h) and BH extent (2.01 vs. 1.35%/h) at a greater rate from 12-24 h of incubation (Table 4.5). Low pH increased NDF disappearance extent at similar rates in cultures, especially A-RF with DC increased NDF disappearance extent faster than other cultures (0.41, 0.38, 0.37, and 0.35 %/h). At high pH, A-RF with HMC increased NDF disappearance extent at a faster rate than other cultures (0.81 vs. 0.78, 0.62, and 0.60 %/h).

Cultures containing A-RF and DC increased BH extent of LA faster than in other cultures (1.60 vs. 1.41, 1.29, and 1.11 %/h) at low pH. At high pH, cultures containing A-RF and HMC increased BH extent of LA faster than in other cultures (2.26 vs. 2.08, 1.95, and 1.77 %/h).

Effects of culture pH, rumen fluid, and starch fermentability on FA profile in cultures after 24 h of incubation

Table 4.6 shows the effects of culture pH, starch fermentability, and rumen fluid type on FA profile in cultures after 24 h of incubation. Overall, compared with high culture pH, low culture pH increased total conjugated linoleic acid (CLA; 0.76 vs. 0.69 g/100 g, $P < 0.001$), and decreased total odd- and branched- chain FA (OBCFA; 2.49 vs. 2.82 g/100 g, $P < 0.001$). Compared with high pH, low pH increased LA (22.5 vs. 17.6 g/100 g, $P < 0.001$), *t*10, *c*12 CLA (0.49 vs. 0.31 g/100 g, $P < 0.001$), and *t*10 18:1 (3.44 vs. 2.72 g/100 g, $P < 0.001$), but decreased *c*9, *t*11 CLA (0.27 vs. 0.38 g/100 g, $P < 0.001$), *t*11 18:1 (4.92 vs. 7.13 g/100 g, $P < 0.001$), and 18:0 (19.6 vs. 22.6 g/100 g, $P < 0.001$).

Compared with U-RF, A-RF decreased LA (19.8 vs. 22.5 g/100 g, $P = 0.009$) and total conjugated FA (0.64 vs. 0.81 g/100 g, $P < 0.001$), but increased total OBCFA (2.71 vs. 2.60 g/100 g, $P < 0.001$). Rumen fluid adaptation had no effect on 18:0 ($P = 0.31$). Compared with U-RF, A-RF decreased *c*9, *t*11 CLA (0.27 vs. 0.37 g/100 g, $P < 0.001$) and *t*10, *c*12 CLA (0.36 vs. 0.44 g/100 g, $P < 0.001$), and increased *t*11 18:1 (6.17 vs. 5.89 g/100 g, $P = 0.004$) and *t*10 18:1 (3.24 vs. 2.92 g/100 g, $P < 0.001$). Compared with DC, HMC decreased LA (19.4 vs. 20.6 g/100 g, $P < 0.001$), 18:0 (20.6 vs. 21.6 g/100 g, $P < 0.001$), and total OBCFA (2.59 vs. 2.72 g/100 g, $P < 0.001$), but increased total conjugated FA (0.75 vs. 0.70 g/100g, $P = 0.01$). Starch fermentability had no effect on *c*9, *t*11 CLA ($P = 0.35$). HMC increased *t*10, *c*12 CLA (0.43 vs.

0.37 g/100 g, $P = 0.005$), *t*11 18:1 (6.26 vs. 5.79 g/100 g, $P < 0.001$) and *t*10 18:1 (3.40 vs. 2.79 g/100 g, $P < 0.001$).

We observed interactions between culture pH and starch fermentability for *t*10, *c*12 CLA and *t*10 18:1. HMC increased *t*10, *c*12 CLA at low pH (0.56 vs. 0.41 g/100 g, $P < 0.001$) compared with DC, but there was no difference at high pH. Starch source also interacted with rumen fluid ($P < 0.001$). Compared with DC, HMC increased *t*10, *c*12 CLA with U-RF (0.50 vs. 0.37 g/100 g, $P < 0.001$), but there was no difference between DC and HMC in cultures with A-RF. We also observed a three-way interaction among culture pH, starch fermentability, and rumen fluid for *t*10, *c*12 CLA ($P < 0.05$). Compared with DC, HMC increased *t*10, *c*12 CLA at low pH with U-RF; however, there was no difference between HMC and DC with A-RF (Table 4.6).

We observed an interaction between starch fermentability and rumen fluid on the concentration of *t*10 18:1 (Table 4.6). While A-RF increased *t*10 18:1 compared with U-RF, the increase was greater in cultures with HMC compared with DC (3.64 vs. 3.16 g/100 g, $P < 0.001$; 2.84 vs. 2.67 g/100 g, $P < 0.05$). We also observed a three-way interaction among culture pH, starch fermentability, and rumen fluid for *t*10 18:1 ($P < 0.05$). Compared with U-RF, A-RF increased *t*10 18:1 by 21% in cultures with HMC at low pH ($P < 0.001$). However, A-RF increased *t*10 18:1 by only 9% in cultures with HMC at high pH ($P < 0.05$).

Changes in t10, c12 CLA and t10 18:1 concentrations in culture over 24 h of incubation

The effects of treatment across time on *t*10, *c*12 CLA and *t*10 18:1 are shown in Figures 4.4 and 4.5, respectively. The appearance rate of *t*10, *c*12 CLA and *t*10 18:1 from 0-12 h and 12-24 h incubation are shown in Table 4.7. Generally, the appearance rate for *t*10, *c*12 CLA was higher during the first 12 h than the second 12 h of incubation (0.022 vs. 0.009 g/100 g/h; Figure

4.5). From 12-24 h of incubation, cultures with A-RF and HMC increased *t*10, *c*12 CLA at a lower rate than other cultures at low pH, and decreased *t*10, *c*12 CLA at high pH. The appearance rate of *t*10 18:1 was higher during the second 12 h than the first 12 h of incubation (0.14 vs. 0.08 g/100 g/h; Figure 4.4). From 12-24 h, cultures with A-RF and HMC increased *t*10 18:1 faster than other cultures at both low and high pH.

DISCUSSION

LA is typically the most abundant unsaturated FA in dairy cow diets, and this is reflected in the concentration of LA in the substrates of our in vitro batch cultures. In the current study, to ensure precise and uniform nutrient composition in culture, we used fewer substrate ingredients than would be found in a typical dairy cow diet. Unsaturated FA are biohydrogenated to SFA by rumen bacteria through several steps and intermediates (Griinari and Bauman, 1999). Among the numerous BH pathways that convert LA to 18:0 in the rumen, the major pathway first involves isomerisation of LA to *c*9, *t*11 CLA and then hydrogenation of *c*9, *t*11 CLA to *t*11 18:1 and 18:0. However, when MFD risk factors exist, including dietary factors and changes in the rumen environment, more LA is biohydrogenated through an alternative pathway producing intermediates including *t*10, *c*12 CLA and *t*10 18:1. The negative effects of *t*10, *c*12 CLA on milk fat synthesis are well-established (Bauman et al., 2011).

Longuski et al. (2009) reported that SCFP supplementation prevented diet-induced MFD, possibly through alteration of rumen microbes and BH pathways. However, the specific effects of SCFP on BH pathways were not determined in that study. Therefore, our objective was to determine the effect of SCFP on BH pathways and the formation of MFD-associated BH intermediates at two starch fermentabilities and two culture pH conditions. By using an in vitro batch culture incubation system, we previously studied the effects of starch fermentability and

culture pH on BH pathways and found that high moisture corn increased *t*10, *c*12 CLA in cultures at low culture pH (Sun et al., 2015). Common incubation time ranges in batch culture studies are less than 24 h (Van Nevel and Demeyer, 1996; Choi and Song, 2005; Troegeler-Meynadier et al., 2006). Due to the stability and specificity of rumen microbes, a new equilibrium status of microbial community would not be established until days after a dietary change (Weimer et al., 2010). Therefore, it is not feasible to test effects of feed additives on BH by adding the supplements directly into culture; especially the potential mechanism is via changing rumen microbial population. To test the effects of diet ingredients and rumen conditions on BH, Vlaeminck et al. (2008) and Zened et al. (2011) utilized rumen inoculum collected from donor cows adapted to treatment diets for 21 or 14 d in their in vitro studies. Therefore, to test our hypothesis, rather than adding SCFP into cultures, we supplemented SCFP in the cows' diet for 28 d to allow rumen microbial population to change and stabilize. In vitro batch cultures were then performed with the rumen inoculum collected from donor cows on the last day of each cow treatment period. We observed numerous interactions among culture pH, starch fermentability, and rumen fluid. We previously reported interactions between culture pH and starch fermentability in vitro (Sun et al., 2015). In our current study, we focused on the effects of interactions among rumen fluid, culture pH and starch fermentability on variables associated with BH of LA.

Supplementing SCFP resulted in differences in rumen fluid including FA profile and pH. Vlaeminck et al., (2008) also reported effects of diet on FA profile of rumen fluid from donor cows. Miller-Webster et al. (2002) reported that SCFP decreased culture pH after 2 h of incubation in a continuous culture system. In our study, rumen fluid for in vitro batch culture was collected 1 h after morning feeding. The pH of A-RF was lower than U-RF (Table 4.3), which

might have been due to enhanced total VFA production in SCFP-supplemented cows (as observed by Miller-Webster et al. [2002]). A-RF contained a higher concentration of *t*10, *c*12 CLA than U-RF. However, the concentration of *t*10, *c*12 CLA was only 0.0049% DM for A-RF. In our recently completed study, we observed that concentrations of *t*10, *c*12 CLA were 0.0043 and 0.0066% DM for cows fed control and MFD inducing diets, respectively (manuscript in preparation). Also, we observed that A-RF decreased concentration of *t*10, *c*12 CLA in culture. A large amount of feed arriving in the rumen following the morning feeding, and subsequent reduction in rumen pH, may have resulted in the difference in the FA composition in rumen fluid between SCFP-supplemented cows and control cows. The present study used two starch sources (DC and HMC) with different fermentabilities to provide 33% starch (DM basis) substrate in vitro with similar FA composition and total FA content. Although it is statistically significant, the total FA content of cultures at different pH levels was less than 1 mg/culture. Changes in individual FA content and concentration of the cultures followed the similar patterns, and we only report the results of FA concentrations and focus on FA associated with BH of LA in this study.

Previous studies have used inconsistent and wide range of pH levels to represent low (5.6 to 6.25) and high (6.4 to 6.78) pH in the investigation of the effects of pH on BH of FA (Fuentes et al., 2011; AbuGhazaleh et al., 2005; Calsamiglia et al., 2002; Troegeler-Meynadier et al., 2013). Low pH inhibits bacteria growth and nutrient digestibility in rumen (Russell and Dombrowski, 1980; Russell and Wilson, 1996), and a pH of 5.8 is often used as a threshold for subacute acidosis for dairy cows (Beauchemin and Yang, 2005; Dohme et al., 2008; Mohammed et al., 2012). Therefore we chose pH levels of 5.8 and 6.2 to represent initial low and high pH, which provided a smaller range compared with other studies. Two pH levels were obtained by

adding citric acid to buffer solution. As discussed in Chapter 2, rumen microbes can utilize citric acid as energy source (Van Soest, 1994), therefore, we can not rule out the effects of culture pH on NDF digestion and BH being attributable to addition of citric acid.

We observed pH fluctuation during incubation, and the average pH was 5.7 and 6.0 for low and high pH cultures across the 24-h incubation period. Troegeler-Meynadier et al. (2013) also reported pH reduction in in vitro cultures, with initial pH of 6.25 and 6.78 and final pH of 5.82 and 6.56, representing low and high pH respectively. Compared with U-RF, A-RF decreased average culture pH, but the difference was less than 0.04 units, and biologically non-significant. Therefore, the effects of A-RF observed in our study were more likely caused by changes in rumen bacteria populations and metabolism, rather than mediated through culture pH change.

NDF disappearance extent provides a measure of the viability of cultures. We observed low overall NDF disappearance extent in the present study, which might have been caused by the high starch concentration (33% DM) in substrates (Sun et al., 2015). Culture pH had the greatest effect on NDF disappearance extent among the factors examined in our experiment. Grant and Mertens (1992) also reported negative effects of low culture pH (< 6.2) on NDF digestibility. Calsamiglia et al. (2002) found that pH 5.7 decreased NDF digestibility compared to pH 6.4. Low pH is detrimental to cellulolytic bacteria, and cells stopped growing in continuous culture when pH was lower than 5.90, 6.0, 6.15, and 5.70 for *Ruminococcus albus*, *Bacteroides succinogenes*, and *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens*, respectively (Russell and Dombrowski, 1980). In contrast to our results, Oba and Allen (2003) reported no effect of HMC on ruminal NDF digestibility in vivo. However, compared with DC, HMC decreased feed intake and led to a lower NDF intake in cows fed HMC, which may have

influenced the effects of starch source on NDF disappearance extent. The effect of SCFP on NDF disappearance extent could be the result of altered rumen microbial metabolism (Miller-Webster et al., 2002), increased microbial protein synthesis (Hristov et al., 2010), and stabilized rumen fermentation (Harrison et al., 1988).

Cellulolytic bacteria (*e.g. Butyrivibrio fibrisolvens*) undertake BH of LA to produce *c*9, *t*11 CLA and *t*11 18:1 (Polan et al., 1964). Similar to NDF disappearance extent, culture pH also influences the BH of LA. Troegeler-Meynadier et al. (2006) reported that low culture pH may inhibit rumen bacteria activity or enzymes involved in the isomerisation of LA and reduction of *trans* 18:1 FA to 18:0. Normally, most dietary LA is biohydrogenated to 18:0 via the intermediates of *c*9, *t*11 CLA and *t*11 18:1. However, changes in the rumen environment and nutrient intake may shift the BH pathway of LA to produce intermediates of *t*10, *c*12 CLA and *t*10 18:1 (Bauman et al., 2011). BH extent provides a measure of LA disappearance in cultures. We observed no difference between HMC and DC on the BH extent of LA, but HMC resulted in more LA being biohydrogenated to *t*10, *c*12 CLA and *t*10 18:1. Cotta (1988) reported that starch-containing medium had different effects on growth rates of selected rumen bacteria species, such as *Bacteroides ruminicola*, *Streptococcus bovis*, and *Butyrivibrio fibrisolvens*. Similar as we observed in Chapter 3, BH extents of current study were relatively lower than previous studies for high starch cultures (Zened et al., 2011 and 2012). Potential explanations include differences in culture pH and sources of LA, which we have discussed in Chapter 3.

Starch in HMC is potentially more available than that in DC. Instead of changing bacterial mass, HMC may have altered the bacterial profile, and influence BH of LA. Our previous study showed that, compared with 33% DC (DM basis), HMC increased the concentration of *t*10, *c*12 CLA at low pH (Sun et al., 2015). We observed similar results in our

current study, but A-RF alleviated the increase in *t*10, *c*12 CLA associated with HMC at low culture pH. Li et al. (2013) tested the effects of SCFP on bacteria during sub-acute rumen acidosis, and suggested that SCFP alleviates the impact of low pH on the rumen bacterial population and increased *Prevotella brevis*, which may play a predominant role in BH (Huws et al., 2011).

Baumgard et al. (2001) reported increasing abomasal infusion doses of *t*10, *c*12 CLA progressively reduced milk fat yield, and 0.016% *t*10, *c*12 CLA (dietary DM basis) markedly inhibited milk fat synthesis. Therefore, reduction of *t*10, *c*12 CLA in cultures by A-RF may decrease risk for MFD. This supports the finding of a meta-analysis that SCFP increases milk fat yield (Poppy et al., 2012). Longuski et al. (2009) supplemented SCFP to dairy cows for 26 d before a fermentable starch challenge in which dry corn was replaced with high moisture corn The fermentable starch challenge tended to decrease milk fat yield in control cows, but had no effect on SCFP-supplemented cows. This result is supported by our findings, which show that rumen fluid from cows supplemented with SCFP reduces the production of *t*10, *c*12 CLA in low pH cultures containing HMC. A-RF increased the disappearance (BH extent) of LA, and increased concentrations of *t*10 18:1 and *t*11 18:1, but had no effect on 18:0 concentration. SCFP may have increased the activity of bacteria that convert LA to *c*9, *t*11 CLA and *t*11 18:1, including *Butyrivibrio fibrisolvens* and *Pseudobutyrivibrio* spp. However, it does not appear to affect the activity of bacteria hydrogenating *t*11 18:1 to 18:0, such as *Butyrivibrio proteoclasticus* (Wallace et al., 2006). This conclusion is supported by the high appearance rate of *t*10 18:1 and low appearance rate of *t*10, *c*12 CLA from 12-24 h of incubation in our study, as well as the increased NDF disappearance extent observed in A-RF cultures with HMC at low pH. Previous studies also reported that SCFP stabilized total rumen bacteria, especially cellulolytic

bacteria, in vitro (Callaway and Martin, 1997; Harrison et al., 1988; Newbold et al., 1995). Mullin et al. (2013) analyzed rumen fluid collected from SCFP-supplemented cows and found no effects of supplementation on tested microbial species. However, the diet ingredients differed between studies, which may have affected rumen microbe populations and fermentation differently (Boguhn et al., 2012), and mitigated the effects of SCFP on rumen bacteria. Furthermore, other uncharacterized microbial species may play an important role in biohydrogenation, but were not tested (Lourenço et al., 2010). These could include uncultured bacteria phylogenetically classified as *Prevotella*, *Lachnospiraceae* incertae sedis and unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* (Huws et al., 2011).

One limitation of our study was that we did not test the microbial biomass in cultures. However, our OBCFA results provided evidence that treatment effects on BH pathway and FA composition in cultures might be caused by alteration of microbial populations. OBCFA are mainly synthesized by rumen microbes, and have previously been used as markers for estimating rumen microbial mass (Vlaeminck et al., 2005). In our study, the concentration (and yield) of total OBCFA was consistent with changes in NDF disappearance extent and BH of LA. There is a high concentration of branched-chain FA in cellulolytic bacteria, including *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, and *Ruminococcus flavefaciens*, compared to a lower concentration in amylolytic bacteria including *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis*, and *Succinomonas amylolytica* (Fievez et al., 2012). As discussed previously, cellulolytic bacteria are the major bacteria associated with BH pathways that produce *c*9, *t*11 CLA and *t*11 18:1. In our study, HMC decreased the concentration of total OBCFA and NDF disappearance extent, increased the concentration of *t*10, *c*12 CLA, and had no effect on BH extent of LA. Therefore, HMC may change BH pathways of LA by decreasing

cellulolytic bacteria, and increasing *t*10, *c*12 CLA-producing bacteria such as *Megasphaera elsdenii* (Kim et al., 2002) and *Propionibacterium acnes* (Devillard and Wallace, 2006). Low pH is detrimental to rumen bacteria (Russell and Dombrowski, 1980), which is consistent with our observed reductions in total OBCFA at low pH, as well as decreased NDF disappearance extent and BH of LA. Compared with U-RF, A-RF resulted in a higher content of total OBCFA under both low and high pH, which is consistent with the NDF disappearance extent and BH of LA results.

CONCLUSION

Our study utilized an in vitro batch culture system to determine the effect of SCFP on FA biohydrogenation under different ruminal and dietary conditions. Low culture pH and HMC decreased NDF disappearance extent and increased the formation of *t*10, *c*12 CLA. Rumen fluid collected from cows supplemented with SCFP (Diamond V Original XPC) increased NDF disappearance extent, and decreased formation of *t*10, *c*12 CLA, especially when combined with HMC at low culture pH. Our study provides information about the mechanism of SCFP prevention of diet-induced MFD; SCFP increased NDF disappearance extent and decreased the formation of *t*10, *c*12 CLA in cultures containing high fermentable starch at low pH. It also supports a previous meta-analysis in which SCFP was shown to increase milk fat yield in dairy cows.

APPENDIX

APPENDIX

Table 4.1. Ingredients and nutrient composition of diet fed to rumen inoculum donor cows[1]

	Diet[2]
Ingredient, % of DM	
Corn silage	22.3
Haylage	12.5
Ground corn	19.7
High moisture corn	8.93
Soybean meal	16.3
Cottonseed with lint	7.25
Soy hulls	7.15
Wheat straw	2.50
Dairy Base VitMin[3]	2.00
Sodium Bi-Carb	0.75
Limestone	0.66
Nutrient composition	
DM, %	57.1
NDF, % of DM	30.5
Starch, % of DM	28.2
CP, % of DM	16.6
Total FA, % of DM	3.41

[1]Average composition of two periods fed to lactating dairy cows (n=6).
[2] Diet for control cows; 14 g/d of Diamond V XPC™ was top dressed for cows supplemented with SCFP.
[3] Vitamin and mineral mix contained 34.1% dry ground shell corn, 25.6% white salt, 21.8% calcium carbonate, 9.1% Biofos, 3.9% magnesium oxide, 2% soybean oil, and < 1% of each of the following: manganese sulfate, zinc sulfate, ferrous sulfate, copper sulfate, iodine, cobalt carbonate, vitamin E, vitamin A, vitamin D, and selenium.

Table 4.2. Ingredient and FA concentrations of substrates used for in vitro batch culture[1]

Item	Substrates	
	DC as starch source	HMC as starch source
Ingredients, % DM		
Alfalfa hay[2]	55	55
DC[3]	45	-
HMC[4]	-	45
Total FA, % DM	3.33	3.36
FA, g/100 g		
16:0	15.1	15.4
18:0	2.23	2.16
*c*9 18:1	22.1	23.4
LA[5]	51.1	50.1
*c*9, *c*12, *c*15 18:3	3.74	3.73
∑ Others	5.70	5.20

[1] Average of two periods, n = 4 per treatment per period.
[2] Alfalfa hay was treated with corn oil (2% DM) to increase total unsaturated FA content in substrate.
[3] DC = dry ground corn.
[4] HMC = high moisture corn.
[4] LA, *c*9, *c*12 18:2.

Table 4.3. pH, total FA content, and FA composition of rumen fluid collected from cows fed a diet with (A-RF) or without (U-RF) supplemented *Saccharomyces cerevisiae* fermentation product[1].

Item	Rumen fluid[2]		SEM	*P*-values[3]
	U-RF	A-RF		
pH	6.11	5.88	0.00	<0.001
Total FA, % DM	3.01	3.09	0.02	0.04
FA, g/100 g total FA				
12:0	0.28	0.28	0.00	NS
14:0	1.32	1.27	0.01	NS
16:0	20.9	21.2	0.06	NS
*c*9 16:1	0.13	0.12	0.00	NS
18:0	49.6	48.4	0.20	0.03
*t*6, -7, -8 18:1	0.46	0.42	0.01	NS
*t*9 18:1	0.31	0.28	0.01	NS
*t*10 18:1	1.14	1.13	0.02	NS
*t*11 18:1	2.29	2.11	0.03	0.03
*t*12 18:1	0.76	0.66	0.03	NS
*c*9 18:1	5.02	5.20	0.04	NS
*c*11 18:1	1.55	1.41	0.01	<0.001
LA[4]	5.08	5.81	0.02	<0.001
*c*9, *t*11 CLA	0.04	0.04	0.00	NS
*t*10, *c*12 CLA	0.08	0.16	0.01	<0.01
*c*9, *c*12, *c*15 18:3	0.51	0.50	0.01	NS
22:0	0.21	0.21	0.01	NS
24:0	0.23	0.22	0.01	NS
∑ Others	6.21	6.38	0.02	0.01
∑ Unknown	3.92	4.22	0.07	0.05
∑ *trans* 18:1	4.95	4.60	0.10	NS
∑ CLA	0.11	0.20	0.01	<0.01

[1] Average of two periods, n=4 per treatment per period

Table 4.3. (cont'd)

[2] A-RF and U-RF were rumen fluid collected at 1 h after morning feeding from experimental cows fed a diet with or without *Saccharomyces cerevisiae* fermentation product, respectively.
[3] *P*-values represent the effect of rumen fluid type. NS, not significant ($P > 0.1$).
[4] LA, *c*9, *c*12 18:2.

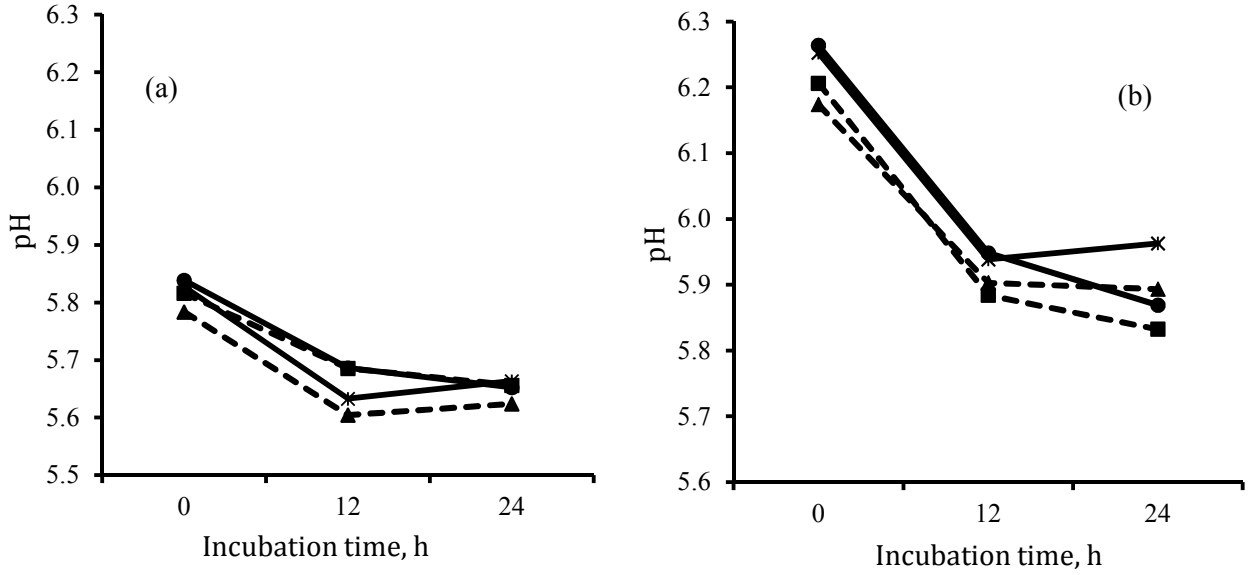

Figure 4.1. pH of cultures incubated with U-RF and DC, U-RF and HMC, A-RF and DC, A-RF and HMC at low pH (a) and high pH (b) over 24 h incubation.
pH of cultures incubated with U-RF and DC (—●—), U-RF and HMC (—✳—), A-RF and DC (- -■- -), A-RF and HMC (- -▲- -) at low pH (a) and high pH (b) over 24 h incubation (SEM = 0.01; interaction among culture pH, starch source and time, $P < 0.01$; interaction among rumen fluid type, starch source and time, $P = 0.10$). U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn.

Table 4.4. pH, NDF disappearance extent, total FA content, and BH extent of LA of in vitro batch cultures after 24 h of incubation[1].

Item	U-RF[2]				A-RF				SEM	*P*-values[3]
	Low pH		High pH		Low pH		High pH			
	DC	HMC	DC	HMC	DC	HMC	DC	HMC		
pH	5.65	5.66	5.87	5.97	5.66	5.63	5.83	5.89	0.01	CpH **, RF **, SS **, CpH × RF *, CpH × SS **, RF × SS *
NDF disappearance extent, %	10.6	8.9	16.8	13.1	10.9	10.1	19.3	15.4	0.44	CpH **, RF **, SS **, CpH × RF *, CpH × SS **
Total FA, mg/culture	25.4	25.6	24.9	25.0	25.0	25.2	24.6	24.8	0.18	CpH **
BH extent of LA[4] , %	29.0	31.7	47.4	44.9	32.3	36.3	47.8	48.9	0.93	CpH **, RF **, CpH × SS **

[1] Values are means of 4 replicates for all variables, except pH value is means of 8 replicates.
[2] U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn.
[3] CpH, effect of culture pH (low pH & high pH); RF, effect of rumen fluid type (U-RF, SCFP un-adapted rumen fluid; A-RF, SCFP adapted rumen fluid); SS, effect of starch source (DC, dry ground corn; HMC, high moisture corn); RF × CpH, interaction of rumen fluid type and culture pH; SS × CpH, interaction of starch source and culture pH; SS × RF, interaction of starch source and rumen fluid type; SS × RF × CpH, interaction of starch source, rumen fluid type and culture pH; *, $P < 0.05$; **, $P < 0.01$.
[4] BH extent of LA, was calculated by subtracting the amount of LA in the cultures at 24 h from the amount at 0 h and dividing by the amount at 0 h.

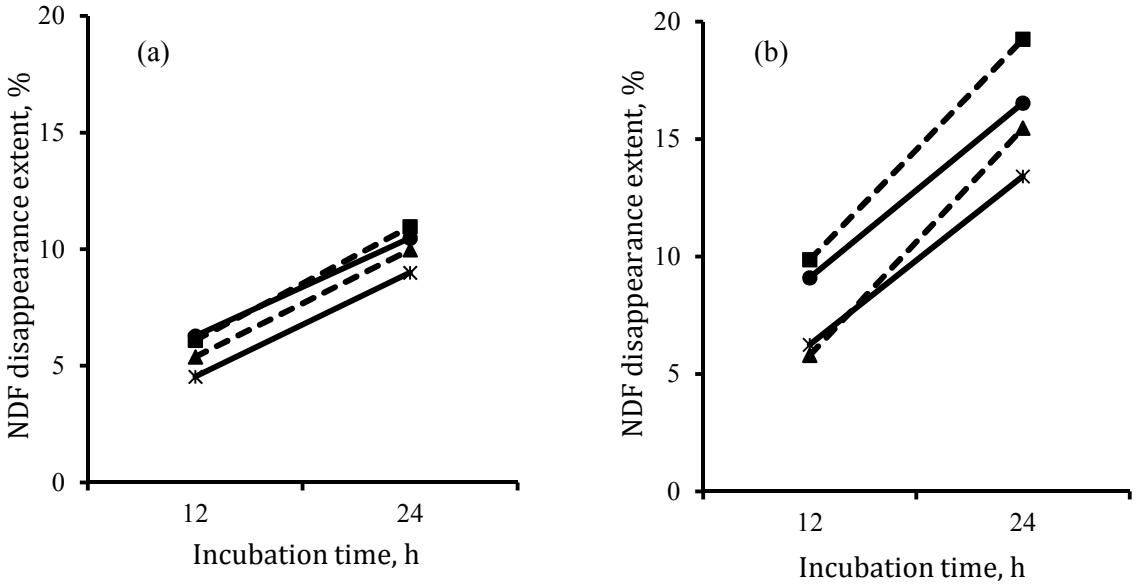

Figure 4.2. NDF disappearance extent of cultures incubated with U-RF and DC, U-RF and HMC, A-RF and DC, A-RF and HMC at low pH (a) and high pH (b) over 24 h of incubation.

NDF disappearance extent of cultures incubated with U-RF and DC ( ), U-RF and HMC ( ), A-RF and DC ( ), A-RF and HMC ( ) at low pH (a) and high pH (b) over 24 h of incubation (SEM = 0.38; interaction among rumen fluid type, culture pH and time, P = 0.04). U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn. The NDF disappearance extent was calculated by subtracting the amount of NDF residue in the cultures at 12 and 24 h from the amount at 0 h and dividing by the amount at 0 h.

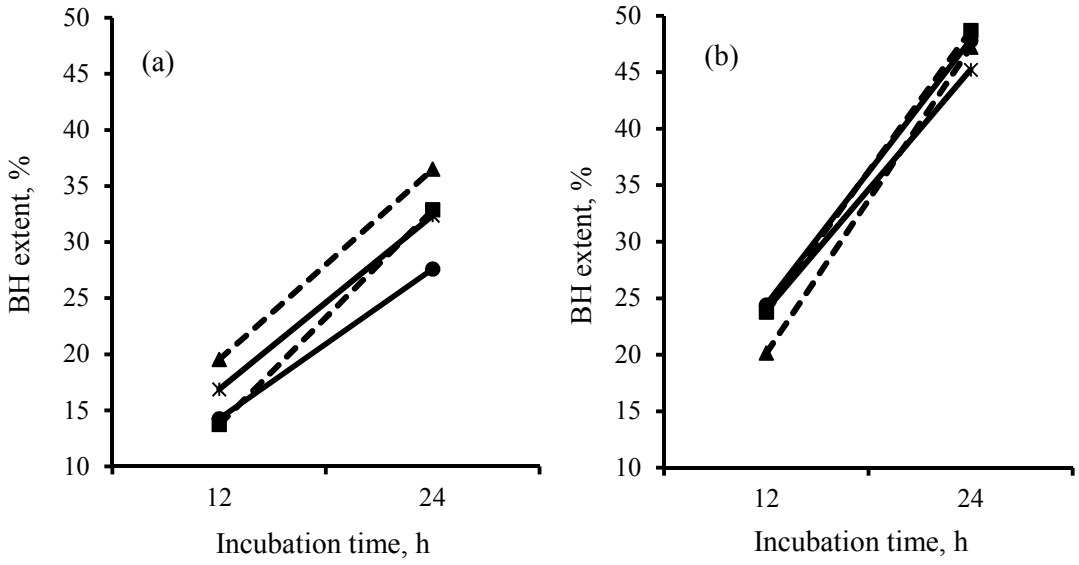

Figure 4.3. BH extent of LA of cultures incubated with U-RF and DC, U-RF and HMC, A-RF and DC, A-RF and HMC at low pH (a) and high pH (b) over 24 h incubation.
BH extent of LA (*c*9, *c*12 18:2) of cultures incubated with U-RF and DC (—●—), U-RF and HMC (—✱—), A-RF and DC (- -■- -), A-RF and HMC (- -▲- -) at low pH (a) and high pH (b) over 24 h incubation (SEM = 1.7; interaction between culture pH and time, $P < 0.01$; interaction between rumen fluid type and time, $P = 0.08$). U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn. The BH extent was calculated by subtracting the amount of LA in the cultures at 12 and 24 h from the amount at 0 h and dividing by the amount at 0 h.

Table 4.5. Increasing rate of NDF disappearance extent and BH extent of LA from 12 to 24 h of incubation[1].

Increasing rate, %/h	U-RF[2]				A-RF			
	Low pH		High pH		Low pH		High pH	
	DC	HMC	DC	HMC	DC	HMC	DC	HMC
NDF disappearance extent	0.35	0.37	0.62	0.60	0.41	0.38	0.78	0.81
BH extent of LA[3]	1.11	1.29	1.95	1.77	1.60	1.41	2.08	2.26

[1] Increasing rates of NDF disappearance extent and BH extent from 12 to 24 h of incubation were calculated by dividing the difference between NDF disappearance extent or BH extent at 12 and 24 h by 12 h.
[2] U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn.
[3] BH extent of LA, was calculated by subtracting the amount of LA in the cultures at 24 h from the amount at 0 h and dividing by the amount at 0 h.

Table 4.6. Concentrations of selected FA of in vitro batch cultures after 24 h of incubation[1].

FA, g/100 g total FA	U-RF[2]				A-RF				SEM	*P*-values[3]
	Low pH		High pH		Low pH		High pH			
	DC	HMC	DC	HMC	DC	HMC	DC	HMC		
12:0	0.62	0.66	0.57	0.57	0.58	0.59	0.52	0.51	0.01	CpH **, RF **, SS*, SS × CpH **
14:0	1.17	1.17	1.23	1.17	1.07	1.07	1.12	1.09	0.01	CpH **, RF **, SS *, SS × CpH **
16:0	17.6	18.0	17.6	17.9	17.7	17.9	17.7	17.9	0.07	SS **
*c*9 16:1	0.24	0.25	0.22	0.23	0.23	0.23	0.20	0.21	< 0.01	CpH **, RF **, SS **
18:0	19.9	19.5	23.0	21.6	19.6	19.5	23.7	22.0	0.24	CpH **, SS **, SS × CpH **
*t*6, -7, -8 18:1	0.26	0.21	0.43	0.36	0.31	0.28	0.46	0.49	0.01	CpH **, RF **, SS **, SS × RF **, SS × RF × CpH *
*t*9 18:1	0.35	0.38	0.49	0.47	0.37	0.40	0.47	0.57	0.01	CpH **, RF **, SS **, SS × RF **, SS × RF × CpH **
*t*10 18:1	3.10	3.36	2.25	2.96	3.24	4.06	2.45	3.22	0.07	CpH **, RF **, SS **, SS × RF **, SS × RF × CpH *
*t*11 18:1	4.86	4.55	6.88	7.26	4.72	5.57	6.70	7.68	0.12	CpH **, RF **, SS **, SS × CpH *, SS × RF **
*t*12 18:1	0.41	0.36	0.68	0.60	0.44	0.43	0.65	0.75	0.02	CpH **, RF **, SS × RF **, SS × RF × CpH *
*c*9 18:1	14.9	15.2	13.8	14.7	14.8	15.3	13.7	14.5	0.09	CpH **, SS **, SS × CpH **
*c*11 18:1	1.24	1.21	1.49	1.46	1.15	1.15	1.43	1.44	0.01	CpH **, RF **
LA[4]	23.4	22.2	17.6	17.7	23.5	20.9	18.0	16.9	0.19	CpH **, RF **, SS **, SS × CpH **, SS × RF **
*c*9, *t*11 CLA	0.30	0.35	0.47	0.39	0.20	0.24	0.36	0.30	0.02	CpH **, RF **, SS × CpH **
*t*10, *c*12 CLA	0.41	0.67	0.34	0.34	0.42	0.46	0.33	0.25	0.03	CpH **, RF **, SS **, SS × CpH **, SS × RF **, SS × RF × CpH *

Table 4.6. (cont'd)

FA, g/100 g total FA	U-RF[2]				A-RF				SEM	*P*-values[3]
	Low pH		High pH		Low pH		High pH			
	DC	HMC	DC	HMC	DC	HMC	DC	HMC		
*c*9, *c*12, *c*15 18:3	1.25	1.29	0.99	1.07	1.25	1.23	1.01	1.02	0.01	CpH **, RF **, SS *, SS × RF **
22:0	0.30	0.31	0.30	0.31	0.29	0.30	0.30	0.31	0.007	SS *
24:0	0.27	0.28	0.25	0.27	0.26	0.28	0.26	0.27	0.01	NS
∑ OBCFA [5]	2.54	2.39	2.79	2.68	2.56	2.46	2.97	2.83	0.02	CpH **, RF **, SS **, RF × CpH **
∑ Others	2.61	2.75	2.87	2.92	2.69	2.78	2.91	2.99	0.02	CpH **, RF **, SS **
∑ Unknown	4.24	5.00	5.69	5.08	4.69	4.89	4.85	4.79	0.32	NS
∑ *trans* 18:1	8.96	8.85	10.7	11.6	9.07	10.7	10.7	12.7	0.17	CpH **, RF **, SS **, SS × CpH *, SS × RF **
∑ CLA [6]	0.70	1.03	0.80	0.73	0.63	0.69	0.69	0.54	0.02	CpH **, RF **, SS *, SS × CpH **, SS × RF **, SS × RF × CpH *

[1] Values are means of 4 replicates for all variables.
[2] U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn.
[3] CpH, effect of culture pH (low pH & high pH); RF, effect of rumen fluid type (U-RF, SCFP un-adapted rumen fluid; A-RF, SCFP adapted rumen fluid); SS, effect of starch source (DC, dry ground corn; HMC, high moisture corn); RF × CpH, interaction of rumen fluid type and culture pH; SS × CpH, interaction of starch source and culture pH; SS × RF, interaction of starch source and rumen fluid type; SS × RF × CpH, interaction of starch source, rumen fluid type and culture pH; *, $P < 0.05$; **, $P < 0.01$, NS, no significant effects of treatments.
[4] LA, *c*9, *c*12 18:2.
[5] OBCFA , odd and branched chain fatty acid, including 13:0, *iso* 13:0, *iso* 14:0, 15:0, *iso* 15:0, *anteiso* 15:0, *iso* 16:0, *anteiso* 17:0.
[6] CLA, conjugated linoleic acids.

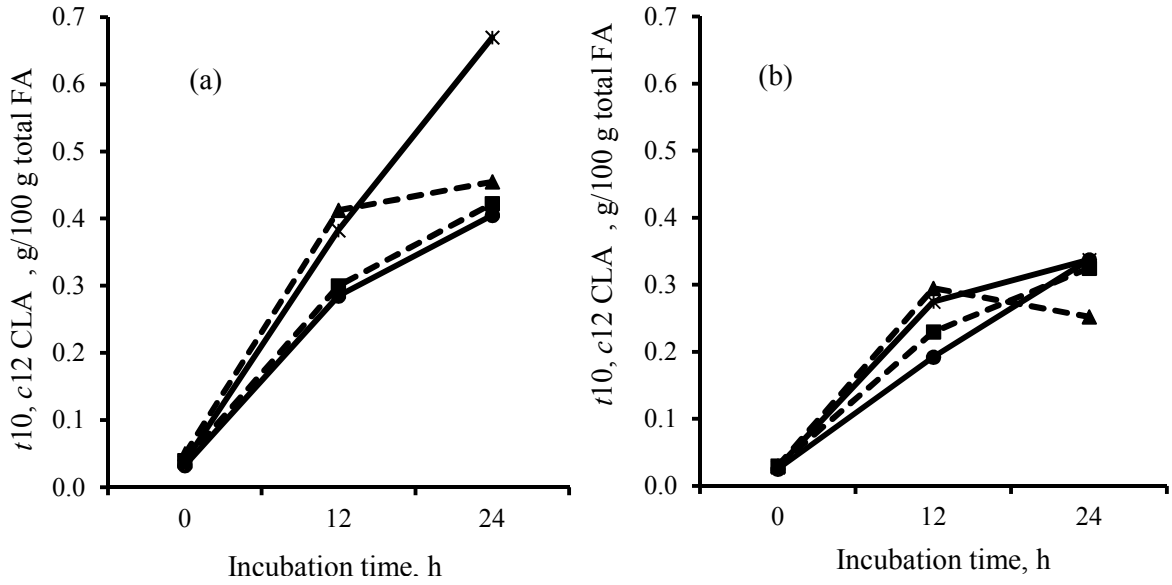

Figure 4.4. Concentration of *t*10, *c*12 CLA of cultures incubated with U-RF and DC, U-RF and HMC, A-RF and DC, A-RF and HMC at low pH (a) and high pH (b) over 24 h of incubation.

Concentration of *t*10, *c*12 CLA of cultures incubated with U-RF and DC (—●—), U-RF and HMC (—✱—), A-RF and DC (- -■- -), A-RF and HMC (- -▲- -) at low pH (a) and high pH (b) over 24 h of incubation (SEM = 0.02; interaction among culture pH, starch source, rumen fluid type and time, $P < 0.01$). U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn.

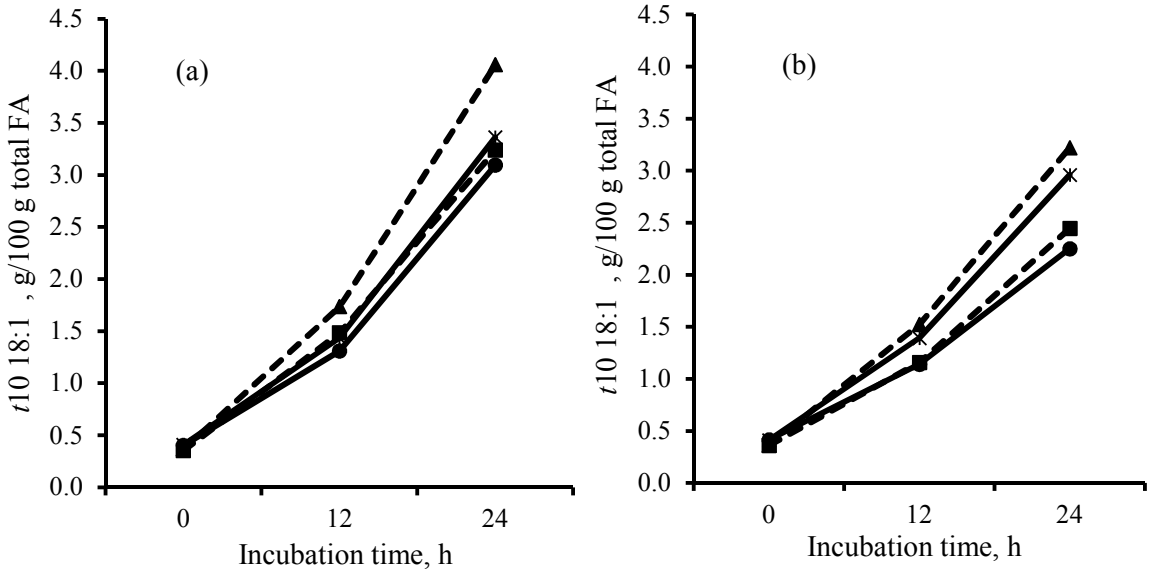

Figure 4.5. Concentration of *t*10 18:1 of cultures incubated with U-RF and DC, U-RF and HMC, A-RF and DC, A-RF and HMC at low pH (a) and high pH (b) over 24 h of incubation.

Concentration of *t*10 18:1 of cultures incubated with U-RF and DC (—●—), U-RF and HMC (—✱—), A-RF and DC (- -■- ·), A-RF and HMC (- -▲- ·) at low pH (a) and high pH (b) over 24 h of incubation (SEM = 0.05; interaction among culture pH, starch source, rumen fluid type and time, $P = 0.02$). U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn.

Table 4.7. Appearance rates of *t*10, *c*12 CLA and *t*10 18:1 from 0 to 12 h and 12 to 24 h of incubation[1].

Appearance rate, g/100 g/h		U-RF[2]				A-RF			
		Low pH		High pH		Low pH		High pH	
		DC	HMC	DC	HMC	DC	HMC	DC	HMC
*t*10, *c*12 CLA	0 to 12 h	0.02	0.03	0.01	0.02	0.02	0.03	0.02	0.02
	12 to 24 h	0.010	0.024	0.012	0.005	0.010	0.004	0.008	-0.004
*t*10 18:1	0 to 12 h	0.08	0.09	0.06	0.08	0.09	0.11	0.07	0.10
	12 to 24 h	0.15	0.16	0.09	0.13	0.15	0.19	0.11	0.14

[1] Appearance rates of *t*10, *c*12 CLA and *t*10 18:1 were calculated as: FA concentration increase in each 12 h incubation period (0 to 12 h or 12 to 24 h) divided by 12 h.
[2] U-RF, rumen inoculum collected from cows fed control diet; A-RF, rumen inoculum collected from cows fed diet supplemented with SCFP; Low pH, cultures started with pH 5.8; high pH, cultures started with pH 6.2; DC, dry ground corn; HMC, high moisture corn.

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CHAPTER 5

PRODUCTION LEVEL OF DAIRY COWS AFFECTS THE EXTENT OF DIET-INDUCED MILK FAT DEPRESSION

INTRODUCTION

Milk fat yield is one of the major components that drive milk income. Reduction in milk fat yield may have a significant impact on the financial income of dairy farms. Previous studies indicated that diet-induced milk fat depression (MFD) is caused by specific biohydrogenation (BH) intermediates (*e.g.* *t*10, *c*12 conjugated linoleic acid, CLA) that pass out of the rumen and subsequently reduce milk fat synthesis in the mammary gland by altering expression of genes involved in fat synthesis (Bauman et al., 2011). Several dietary and rumen environmental factors may affect rumen BH pathways and increase risk for MFD, including high dietary starch content, high starch fermentability, high intakes of unsaturated FA (UFA), and low rumen pH (Trogegeler-Meynadier et al., 2006; Fuentes et al., 2011; Zened et al., 2011; Sun et al., 2014 and 2015).

Additionally, previous studies have shown that cows at different production levels might respond differently to the same diet (Voelker et al., 2002; Boerman et al., 2015a). Therefore, production level may impact risk of diet-induced MFD of dairy cows. There is limited research determining the interaction between production level and diet-induced MFD; moreover, results have been inconsistent and mechanisms not resolved. Bradford and Allen (2004) found that milk fat response to different starch sources differed by production level, with lower producing cows exhibiting a greater reduction in milk fat concentration when fed a more highly fermentable starch source, compared with higher producing cows. Although not determined in this study, it was proposed that higher producing cows may have been able to maintain higher rumen pH and, therefore, experienced a smaller impact on BH pathways due to a better ability to absorb VFA

and stabilize the rumen environment (Voelker et al., 2003). However, Rico et al. (2014) reported that low producing cows were able to maintain milk fat concentration while high producing cows exhibited a decrease in milk fat concentration when fed a diet supplemented with calcium salts of palm FA. The authors reasoned that an increased rumen passage rate in high producing cows resulted in more FA intermediates associated with MFD leaving the rumen, and thus, caused greater MFD.

We have previously used an in vitro batch culture system to test the effects of different dietary factors and culture pH on BH of unsaturated FA. We found that culture pH had the greatest impact on BH pathways, which increased the accumulation of *t*10, *c*12 CLA. Increasing dietary unsaturated FA, or starch content and starch fermentability at low pH resulted in greater accumulation of *t*10, *c*12 CLA than at high pH (Sun et al., 2014 and 2015). In the current study, our objective was to determine the interactive effects between diet and production level on risk of diet-induced MFD in dairy cows. Our hypothesis was that lower producing cows will have a greater response to diet-induced MFD, exhibiting less stable rumen pH and more pronounced MFD than higher producing cows.

MATERIALS AND METHODS

Animal Housing and Care

The Institutional Animal Care and Use Committee at Michigan State University approved all experimental procedures. All cows were housed in tie-stalls throughout the entire experiment at the Michigan State University Dairy Teaching and Research Center. Cows were fed once daily (1200 h) at 115% of expected intake and milked twice daily (0400 and 1500 h). Access to feed was blocked from 1000 to 1200 h to allow for collection of orts and offering of new feed. Water

was available ad libitum in each stall and stalls were bedded with sawdust and cleaned twice daily.

Design and Treatments

Thirty-two mid- and late-lactation multiparous Holstein cows (192 ± 93 DIM; mean ± SD; 14 rumen cannulated and 18 non-cannulated) with a wide range in milk yield (25 to 60 kg/d; 41 ± 12 kg/d; mean ± SD) were used in a crossover design experiment with 28-d periods. Cows were fed a common diet during a 14-d covariate period. Cows were blocked by cannulation and assigned randomly to treatment sequence within level of milk production.

Treatments consisted of: 1) a control diet (CON) containing high forage and low concentrate, and 2) a diet designed to induce MFD (MFDI) containing low forage, high concentrate, and supplemented UFA. The ingredient and nutrient composition of the diets fed as TMR are described in Table 5.1. Diets were formulated to meet requirements of the average cow in the group according to NRC (2001) recommendations. Nutrient compositions of diets were within the range of typical commercial farm rations in the Midwest region of the United States. DM composition was determined twice per week for forages and diets were adjusted when necessary.

Data and Sample Collection

Production data was collected during the last 3 d of the covariate period. Samples and data for production variables were collected from both rumen-cannulated and non-cannulated cows from d 22 to 26 of each treatment period. From rumen-cannulated cows, only, samples and data for total tract nutrient digestibility and rumen fermentation were collected from d 22 to 26. Rumen evacuations were performed on d 27 and 28 to determine rumen nutrients pool and rumen FA BH responses.

During d 22 to 26, samples of diet ingredients (0.5 kg) and orts (12.5%) from each cow were collected daily and composited by period for analysis. Milk yield was recorded and two milk samples were collected at each milking. One aliquot was collected with preservative (bronopol tablet; D&F Control System, San Ramon, CA) in a sealed tube and stored at 4°C for milk components analysis. The second aliquot was stored without preservative at -20°C until it was composited for each cow by period for FA composition analysis. Fecal (~ 400 g), rumen fluid (~ 200 ml), and plasma (~ 15 ml) samples were collected every 15 h over 5 d, resulting in 8 samples per cow per period that represented every 3 h of a 24-h period to account for diurnal variation. Feces were hand-grabbed and stored in a sealed plastic cup at -20°C. Rumen fluid was collected from 4 consistent locations within the rumen and combined. Rumen pH was tested using a portable pH meter (ATI Orion, Boston, MA) and samples were stored in a sealed specimen cup at -20°C until they were composited for VFA and lactate content analysis for each cow by period. Blood was collected by coccygeal venipuncture into two evacuated tubes containing potassium EDTA as an anticoagulant or potassium oxalate as an anticoagulant and sodium fluoride as a glycolytic inhibitor. Blood was stored on ice until centrifugation at 2,000 × g for 15 min at 4°C (within 30 min of sample collection). Plasma was transferred to microcentrifuge tubes and stored at -20°C until being composited for each cow by period for analysis.

BW was recorded three times per wk, after PM milking, throughout treatment periods (d 8 to 28) to determine change in BW gain. Three trained investigators determined BCS on a 5-point scale (in 0.25 point increments; Wildman et al., 1982) on d 26 each period.

Rumen contents were manually evacuated through the rumen cannula 6 h after feeding (1800 h) on d 27 and 40 h later at 2 h before feeding (1000 h) on d 28 of each treatment period.

Total mass and volume of rumen contents were recorded. To ensure accurate sampling, every tenth handful of digesta (10%) was separated as a subsample during the evacuation. Subsamples were strained through a nylon screen (1-mm pore size) in order to separate solid and liquid phases. Both phases were weighed and sampled (350 ml) into sealed plastic cups to determine nutrient pool size. All rumen content samples were stored at -20°C until freeze-drying and composited by evacuation by cow per period.

Sample Analysis

Diet ingredients, orts, and feces were dried at 55°C in a forced-air oven for 72 h to determine DM concentration. Dried samples from each period were ground through a Wiley mill (1 mm screen; Arthur H. Thomas, Philadelphia, PA). Fecal samples were composited on a DM basis for each cow by period. Rumen solid and fluid samples from evacuations were lyophilized in a lyopholizer (FTS Systems, Toronto, Canada), ground with Wiley mill (1 mm screen; Arthur H. Thomas, Philadelphia, PA), and composited based on the original DM ratio of solid and fluid fractions of each evacuation by cow per period. Ground feed ingredients were analyzed for starch (Hall, 2009), crude protein (AOAC International, 2000; method 990.03), and NDF with heat-stable α-amylase and sodium sulfite (Van Soest et al., 1991) by Cumberland Valley Analytical Services Inc. (Hagerstown, MD). Ground orts, composited fecal samples, and composited rumen content samples were analyzed for NDF and starch using the same methods. The content of FA in feed ingredients, orts, feces, and rumen contents were determined as described by Lock et al. (2013). Diurnal rumen fluid samples were composited for each cow by period and were analyzed for major VFA concentrations as described by Harvatine et al., (2002).

Indigestible NDF was used to determine fecal mass and total tract nutrient digestibility (Cochran et al., 1986), and was estimated as NDF residue after 240 h of in vitro fermentation

(Goering and Van Soest, 1970). In vitro flasks were re-inoculated at 120 h to ensure viability of microbes.

Milk samples stored with preservative were analyzed for contents of fat, true protein, and lactose with mid-infrared spectroscopy (AOAC, 1900, method 972.160) by the Michigan Dairy Herd Improvement Association (Universal Lab Services, Lansing, mi). Milk yield and composition for each milking were used for calculating yields of 3.5% FCM, ECM, milk components, and milk energy. Values from each milking were summed to calculate daily yields and then averaged for each collection period. Individual milk samples collected without preservative were composited for each cow by period based on milk fat yield (d 22-26 of each treatment period). FA composition of milk fat was determined as described preciously in our lab (Lock et al., 2013). Milk fat yield and individual FA concentrations were used to calculate FA yields. Individual FA yields on a mass basis were corrected for glycerol content and other milk lipid classes using the molecular weight of each FA (Piantoni et al., 2013).

Plasma metabolite concentrations were analyzed using commercial kits. Bovine Insulin ELISA was used to analyze plasma insulin concentration by solid phase two-site enzyme immunoassay (Mercodia, Uppsala, Sweden). The glucose oxidase method was used to quantify glucose concentration (PGO Enzyme Product No. P7119; Sigma Chemical Co.). Samples for plasma glucose and insulin concentrations were analyzed in duplicate, and allowed a maximum CV of 5% between duplicates.

Calculations

Rumen nutrients pool sizes (kg) were calculated by multiplying the total DM mass of rumen contents and the concentration of each nutrient component. Turnover rate was calculated

as (%/h) = 100 × (intake of component/ rumen pool of component)/24, as described by Kammes and Allen (2012).

Energy partitioning was determined using milk component concentrations, BW, and BCS from each treatment period. Energy outputs expended for milk production, maintenance, and body tissue gain were calculated for each treatment period. According to NRC (2001), milk energy output (Mcal/d) was calculated with an adjustment in the coefficient to account for the difference between true protein and crude protein: Milk energy output (Mcal/d) = [9.29 × fat (kg) + 5.63 × true protein (kg) + 3.95 × lactose (kg)], where yields of milk components were the average for each cow per treatment period. Maintenance energy was calculated as 0.08 times metabolic BW, where metabolic BW was estimated as $BW^{0.75}$, and BW was the average of each cow during each treatment period. Energy for body tissue gain (Mcal/d) was calculated as [(2.88+1.036 × BCS) × ΔBW] (NRC, 2001), where ΔBW was daily BW change (kg/d) and was estimated for each cow by linear regression of BW within each treatment period after two iterations of removing outliers. Dietary energy concentration was calculated based on total energy for milk output, maintenance, and body tissue gain per kg of DMI for each cow as described by Boerman et al. (2015).

Energy partitioning (%) for each energy fraction was calculated as the ratio of each energy fraction to total energy: % to milk output, maintenance, or body tissue gain = [milk energy output, maintenance energy, or body tissue gain energy / (milk energy output + maintenance energy + body tissue gain energy) × 100] %.

Statistical Analysis

All data were analyzed by using the fit model procedure in JMP (version 12.1.0; SAS Institute Inc., Cary, NC), with the following model:

$Y_{ijk} = \mu + C_i[B_l] + P_j + T_k + B_l + T_k \times P_j + T_k \times B_l + pMY + T_k \times pMY + e_{ijk}$, where Y_{ijk} = dependent variable, μ = overall mean, $C_i[B_l]$ = random effect of cow nested within block (i = 1 to 30), P_j = fixed effect of period (j = 1 to 2), T_k = fixed effect of treatment (k = 1 to 2), B_l = fixed effect of block (l = 1 to 2), pMY = preliminary milk yield (linear and quadratic), $T_k \times P_j$ = interaction between treatment and period, $T_k \times B_l$ = interaction between treatment and block, $T_k \times$ pMY = interaction between treatment and preliminary milk yield (linear and quadratic), e_{ijk} = residual error. Rumen parameters and digestibility were analyzed with the same model without block. Both linear and quadratic effects of preliminary milk yield and the interaction between treatment and preliminary milk yield were added to evaluate responses to treatment by level of preliminary milk yield. The quadratic effect of preliminary milk yield and the interaction between it and treatment were not significant ($P > 0.20$) and were removed from the model. The interactions were removed from the model when $P > 0.20$, and the reduced model was used to determine the treatment effect. For informational purposes, the interaction between treatment and preliminary milk yield was included in results tables. The normality of residuals was checked with box plots and the homogeneity of variances with plots of residuals against predicted values. Data was transformed when necessary, and this is noted in results tables. Main effects were declared significant at $P \leq 0.05$, and tendencies were declared at $P \leq 0.10$. Interactions were declared significant at $P \leq 0.10$, and tendencies were declared at $P \leq 0.15$. Overall, the interaction between treatment and period was not significant. The interaction between treatment and block was significant for some variables. The responses of cannulated and non-cannulated cows to treatment are not shown separately because they were similar in tendency. Pearson

correlation coefficients were determined for some variables. All data are presented as least square means plus or minus the standard error of the mean unless otherwise specified.

Two cows were excluded from the dataset (one rumen cannulated and one non-cannulated) due to 50% or greater reduction in of milk yield from period 1 to period 2. Data collected from one non-cannulated cow in period 2 was removed from the database due to mastitis in period 2.

RESULTS

Diets

Ingredient and nutrient composition of treatment diets are shown in Table 5.1. By altering the ratio of forage to concentrate and supplementing FA, we achieved two treatment diets that differed in their contents of NDF, forage NDF, starch, and FA, and all differences were less than or equal to 6%. DM The CON diet contained 34% NDF, 21% forage NDF, 23% starch, and 2.9% FA, while the MFDI diet contained 28% NDF, 19% forage NDF, 29% starch, and 4.3% FA.

Production Responses

Treatment interacted with preliminary milk yield to affect DMI (interaction, $P < 0.01$; Table 5.2). As shown in Figure 5.1, compared with CON, MFDI increased DMI in low producing cows, but decreased DMI in high producing cows. Compared with CON, MFDI increased intakes of 16:0, 18:0, *c*9 18:1, *c*9, *c*12 18:2, total FA, and rumen unsaturated FA load (RUFAL) by 88, 52, 86, 34, 46, and 42%, respectively (all $P < 0.001$, Table 5.3). Overall, CON increased intake of *c*9, *c*12, *c*15 18:3 ($P < 0.001$) compared with MFDI, and the increase was greater in higher producing cows than in lower producing cows (interaction, $P < 0.001$). Overall, compared with CON, MFDI decreased 3.5% FCM (1.4 kg/d, $P = 0.05$), milk fat concentration (0.38%, $P < 0.01$), and milk fat yield ($P = 0.05$; Table 5.2). Compared with CON, MFDI

decreased 3.5% FCM yield in higher producing cows, but did not affect 3.5% FCM yield in lower producing cows (interaction, $P < 0.001$; Figure 5.2). Figures 5.3 shows the relationship between preliminary milk yield and milk fat concentration, and Figure 5.4 shows the relationship between preliminary milk yield and milk fat yield. Compared with CON, MFDI decreased both yield and concentration of milk fat in higher producing cows but did not influence yield and concentration of milk fat in lower producing cows (both interaction, $P < 0.10$). The interaction between production level and milk fat yield indicated that response of milk fat synthesis to MFDI was negatively associated with preliminary milk yield, meaning higher producing cows exhibited a greater reduction in milk fat synthesis than lower producing cows. MFDI increased the yields of milk (1.3 kg/d, $P = 0.01$), milk protein (0.05 kg/d, $P < 0.01$), and milk lactose (0.07 kg/d, $P = 0.01$) compared with CON, but did not influence DMI, ECM, or feed efficiency (ECM/DMI). Compared with CON, MFDI increased milk protein yield in higher producing cows, but did not influence milk protein yield in lower producing cows (interaction, $P = 0.01$; Figure 5.5). Compared with CON, MFDI increased change in BCS (0.07, $P = 0.02$), but had no effect on BW, BCS, or change in BW.

Milk FA Profile and Yields

Generally, MFDI decreased concentrations of FA formed by de novo synthesis and mixed sources (both $P < 0.01$) and increased the concentration of total FA derived from preformed sources ($P < 0.01$, Table 5.4). Treatment interacted with preliminary milk yield for concentrations of preformed FA (interaction, $P < 0.05$). The relationship between preformed FA and preliminary milk yield followed the same pattern as DMI and preliminary milk yield. Compared with CON, MFDI decreased concentrations of most individual FA with carbon lengths of less than 18 (all $P < 0.01$). However, treatment did not influence concentrations of *c*9

14:1 and *c*9 16:1. MFDI increased concentrations of most individual FA with carbon lengths greater than or equal to 18 (all $P < 0.01$), but decreased the concentration of *c*9, *c*12, *c*15 18:3 compared with CON. Treatment did not affect concentrations of 18:0. Notably, MFDI increased concentrations of *t*10 18:1 by 2.5-fold ($P < 0.01$), *t*9, *c*11 CLA by 1-fold ($P < 0.01$), and *t*10, *c*12 CLA by 10-fold (0.011 vs. 0.001 g/100 g, $P < 0.01$). Figure 5.8 illustrates the relationship between concentration of *t*10, *c*12 CLA and preliminary milk yield, in which the concentration of *t*10, *c*12 CLA was relatively consistent in CON but increased in MFDI with increasing preliminary milk yield (interaction, $P < 0.10$). This interaction indicates that higher producing cows exhibited a greater increase in concentration of *t*10, *c*12 CLA than did lower producing cows when treated with MFDI.

Overall, treatment did not affect yield of preformed FA (Table 5.5). Compared with CON, MFDI decreased yields of de novo-synthesized FA (64 g/d, $P < 0.01$; Table 5.5), FA from mixed sources (44 g/d, $P < 0.01$), and OBCFA (4.3 g/d, $P < 0.01$). We also observed interactions between treatment and preliminary milk yield for these FA, in which the disparity between MFDI and CON increased with increasing milk yield (interaction, all $P < 0.05$; Figures 5.6 and 5.7). All interactions indicate that higher producing cows fed MFDI experienced a greater extent of reduction in FA synthesis than lower producing cows.

Calculated Energy Values, Energy Partitioning and Plasma Parameters

Table 5.6 shows the calculated energy outputs by cows fed CON and MFDI. Compared with CON, MFDI did not affect apparent NEL intake, milk energy output, body energy gain, or maintenance energy. We also observed no effects of treatment on the partitioning of total energy toward milk, body tissue gain, or maintenance.

In general, MFDI tended to increase concentration of plasma glucose (1.1 mg/dL, $P = 0.06$; Table 5.7) and increased insulin (0.29 ug/L, $P < 0.01$). As shown in Figure 5.9, with increasing preliminary milk yield, glucose concentration was consistent in cows fed MFDI ($R^2 = 0.01$; $P = 0.68$), but decreased in cows fed CON ($R^2 = 0.42$; $P < 0.01$).

Rumen pH and VFA Concentrations

Effects of treatment diets on rumen pH of rumen-cannulated cows are shown in Table 5.8. In general, MFDI decreased mean rumen pH (0.13, $P < 0.001$) and minimum rumen pH (0.16, $P = 0.001$), and increased rumen pH range (0.20, $P = 0.01$). The difference between MFDI and CON cows decreased with increasing preliminary milk yield, although mean rumen pH was consistent in MFDI cows (Figure 5.10). Higher producing cows exhibited increased pH range on MFDI compared with CON, but the treatment did not influence the rumen pH range of lower producing cows (interaction, $P = 0.03$; Figure 5.11). Overall, treatment had no effect on maximum rumen pH. However, compared with CON, MFDI decreased maximum rumen pH in lower producing cows, but increased maximum rumen pH in higher producing cows (interaction, $P = 0.01$; Figure 5.12).

Table 5.9 illustrates the effects of treatment on rumen VFA concentrations of rumen-cannulated cows. Overall, treatment did not affect total rumen VFA concentration. However, MFDI decreased the molar proportion of acetate (4.4 mol/100 mol, $P < 0.001$), and increased molar proportion of propionate (3.4 mol/100 mol, $P < 0.001$) and the ratio of acetate to propionate ($P < 0.001$). Propionate difference between MFDI and CON was greater in higher producing cows than in lower producing cows (interaction, $P < 0.10$; Figure 5.13). Figure 5.14 shows the relationship between butyrate and preliminary milk yield. Butyrate was similar in CON cows across production levels, but butyrate increased in lower producing MFDI cows and

decreased in higher producing MFDI cows. Treatment also affected molar proportions of other VFA, but the extent of these changes was biologically minor.

Nutrients Digestion and Rumen FA Pool

In general, treatment had no effect on DM digestibility, but MFDI decreased digestibility of NDF (11.5%, $P < 0.001$) and total FA compared with CON (5.3%, $P < 0.01$; Table 5.10). Compared with CON cows, MFDI cows exhibited lower NDF intake (1.24 kg/d, $P < 0.001$) and higher total FA intake (0.38 kg/d, $P < 0.001$). Although treatment did not affect the rumen pool of NDF, MFDI decreased the turnover rate of NDF (0.54%/h, $P < 0.05$). CON resulted in higher NDF intake than MFDI across production levels, but the difference increased with increasing milk yield (interaction, $P < 0.01$). MFDI increased the rumen pool of FA by 25% ($P < 0.001$) and the rumen turnover rate of total FA by 20% ($P < 0.01$). The difference in FA intake between MFDI and CON decreased with increasing preliminary milk yield (interaction, $P < 0.05$). With increasing preliminary milk yield, MFDI decreased FA turnover rate, while CON resulted in consistent FA turnover rate (interaction, $P < 0.10$). Although treatment did not affect iNDF intake, overall, we did observe that MFDI increased iNDF intake in lower producing cows but decreased iNDF intake in higher producing cows, compared with CON (interaction, $P < 0.01$). Compared with CON, MFDI decreased rumen wet mass (3.9 kg, $P < 0.05$), but treatment did not affect rumen mass DM or volume.

Table 5.11 shows the effects of treatment on rumen pool size of individual FA of rumen-cannulated cows. Although MFDI decreased *c*9, *c*12, *c*15 18:3 (1.53 g, $P = 0.02$) compared with CON, it increased both total PUFA (25 g, $P = 0.01$) and *c*9, *c*12 18:2 (26 g, $P = 0.01$), which comprised over 80% of total PUFA. Compared with CON, MFDI increased rumen pool of *t*10, *c*12 CLA and *t*10 18:1 by 24 and 58%, respectively (both $P < 0.05$), but did not affect *t*11 18:1 or

*c*9, *t*11 CLA. Compared with CON, MFDI increased total SFA (63 g, $P < 0.001$) and increased 16:0 and 18:0 by 44 and 12%, respectively (both $P < 0.05$). Treatment also affected total OBCFA in the rumen, with OBCFA being lower in MFDI cows than in CON cows (1.2 g, $P < 0.001$). MFDI also decreased the ratio of total *iso* FA and total *aiso* and linear odd-chain FA compared with CON (0.48 vs. 0.43, $P = 0.001$).

Pearson Correlation Coefficients

Using Pearson correlation analysis, we assessed the relationship among variables. Table 5.12 shows the correlation coefficients among production variables, plasma insulin and metabolites, and specific milk FA concentrations for all cows (n = 30). Milk yield was negatively correlated with plasma glucose concentration ($P < 0.01$) and plasma insulin concentration ($P < 0.01$), and weakly positively correlated with the concentration of *t*10, *c*12 CLA in milk ($P < 0.05$). Milk fat yield was negatively associated with concentrations of plasma glucose ($P < 0.01$) and plasma insulin ($P < 0.01$), and weakly positively associated with the concentration of *t*10 18:1 in milk ($P < 0.05$). Milk fat concentration was negatively correlated with the concentrations of *t*10, *c*12 CLA ($P < 0.01$) and *t*10 18:1 in milk ($P < 0.01$).

Table 5.13 shows the correlation coefficients between milk fat concentration and yield, specific milk FA, the rumen pool of *t*10, *c*12 CLA, FA intake, and rumen pH of rumen-cannulated cows (n = 13). Milk fat yield was weakly positively correlated with intake of *c*9, *c*12 18:2 ($P = 0.01$) and total RUFAL ($P = 0.03$). Milk fat concentration was positively correlated with rumen minimum pH ($P = 0.004$), negatively correlated with concentrations of *t*10, *c*12 CLA and *t*9, *c*11 CLA in milk (both $P < 0.001$), rumen *t*10, *c*12 CLA pool ($P < 0.001$), rumen maximum pH ($P = 0.009$), rumen pH range ($P < 0.001$; Figure 5.15), and rumen propionate molar proportion ($P < 0.001$). However, milk fat concentration was not associated with intake of

*c*9, *c*12 18:2, total RUFAL, or mean rumen pH. Milk *t*10, *c*12 CLA concentration was negatively correlated with rumen minimum pH ($P = 0.005$) and positively correlated with milk *t*9, *c*11 CLA concentration ($P < 0.001$), rumen *t*10, *c*12 CLA pool ($P < 0.001$; Figure 5.17), and rumen pH range ($P = 0.002$). Similarly, milk *t*9, *c*11 CLA concentration was negatively correlated with rumen minimum pH ($P = 0.01$) and positively correlated with the rumen pool of *t*10, *c*12 CLA ($P < 0.001$), rumen pH range ($P < 0.001$), and rumen maximum pH ($P = 0.02$). The rumen *t*10, *c*12 CLA pool was positively correlated with rumen pH range ($P = 0.001$) and rumen minimum pH ($P < 0.001$; Figure 5.16).

DISCUSSION

Diet-induced MFD can significantly impact the profit of dairy producers. Because of research conducted over past 20 years, we better understand risk factors for and mechanisms of diet-induced MFD (Bauman et al., 2011). However, we are still unsure of the influence of milk production level on response of dairy cows to diet-induced MFD. Previous studies have reported conflicting results in this area of inquiry. Bradford and Allen (2003) studied the effects of dietary starch fermentability in a high-starch diet (> 32% starch) on dairy cow productivity. They found no treatment effect on milk fat yield, however high moisture corn decreased milk fat concentration in low producing cows but not in high producing cows. Rico and Harvatine (2014) reported opposing results in which a diet supplemented with 2.3% Ca-salts of palm FA reduced milk fat concentration in high producing cows, but not low producing cows. Ca-salts of palm FA supplementation did not affect milk fat yield in this study (Rico and Harvatine, 2014). However, it is worth noting that the treatment diets in the two studies described above were not designed to induce MFD or to test the interaction between MFD and production level. The mechanism of milk fat reduction in these studies might differ due to differing nutrient compositions of the

treatment diets. Therefore, our objective was to determine the impact of production level on response to diet-induced MFD. MFD can occur when dairy cows are fed diets high in concentrate and low in forage or diets supplemented with PUFA (Bauman and Griinari, 2003). Several researchers have successfully induced MFD by increasing dietary starch, adding plant or fish oil to diets, or by combining these approaches (He and Armentano, 2011; Rico and Harvatine, 2013; Ramirez Ramirez et al., 2015; Ma et al., 2015). In our study, instead of adding a large amount of starch (more than 10%) or PUFA (more than 2%) to induce MFD, we made only minor alterations (< 6% difference in major nutrients; 1% difference in total FA) in order to obtain our treatment diets (CON vs. MFDI).

Previous studies reported that high-starch diets increased DMI compared with low-starch diets (Oba and Allen, 2003a; Boerman et al., 2015a). In our experiment, MFDI contained a higher starch content than CON, but had no overall effect on DMI. However, MFDI increased DMI in lower producing cows but decreased DMI in higher producing cows, compared with CON. Similarly, Boerman et al., (2015b) also reported that a high-starch diet increased DMI in lower producing cows, but decreased DMI in higher producing cows compared with a high-fiber, high-fat diet. High-forage diets often decrease DMI due to the effect of increased rumen physical fill. Therefore, substituting concentrate for forage can increase DMI until rumen metabolic fuel is the limiting factor (Allen, 2000). In our study, MFDI had no effect on rumen DM pool and rumen mass volume, but did have a minor effect on the rumen wet matter pool. We observed an increase in molar percentage of propionate due to MFDI, and this increase was greater in higher producing cows than in lower producing cows. This suggests that higher propionate may be the primary factor limiting DMI of higher producing cows. High propionate can stimulate fuel

oxidation in the liver and cause satiety sooner, especially when plasma insulin concentration is high (Allen, 2014).

In the current study, we observed increases in yield of milk, milk lactose, and milk protein due to MFDI. Increasing dietary starch typically provides more glucose precursors and increases milk production (Boerman et al., 2015b). Previous studies have also reported increased milk protein yield associated with high concentrate diets (Boerman et al., 2015b; Oba and Allen, 2003ab). Elevated insulin may have increased milk protein synthesis in these instances by increasing the efficiency of N utilization by cows fed high-starch diets (Winkelman and Overton, 2013). Additionally, although it was not determined in the current study, high-starch diets may stimulate rumen microbial protein synthesis and increase milk protein yield (Oba and Allen, 2003ab), especially in higher producing cows with increased starch intake. A large portion of OBCFA in milk originate from rumen bacteria, and Vlaeminck et al. (2006) suggested that milk OBCFA could be used as a tool to predict rumen bacteria populations and flow from rumen. However, this relationship may not apply in our study due to the observed MFD. Gene expression of enzymes responsible for triglyceride synthesis in the mammary gland is inhibited during diet-induced MFD, which limits free FA, including OBCFA, incorporation to the glycerol backbone (Bauman et al., 2011).

The primary objective of our study was to test the interaction between production level and dietary treatments. As expected, cows experienced reductions in milk fat concentration and yield in response to MFDI. However, in contrast to our hypothesis, higher producing cows were more sensitive to the effects of MFDI compared to lower producing cows. We also observed that MFDI resulted in greater reductions in the yields of both de novo-synthesized and mixed-source FA in higher producing cows than in lower producing cows. However, we did not observe

treatment effect on yield of FA from preformed sources. Because the gene expression of lipid synthesis enzymes, including FA synthase, acetyl-CoA carboxylase, and lipoprotein lipase (Bauman et al., 2011), is depressed during diet-induced MFD, it is common to observe decreases in yields of all sources of milk FA during MFD (Ramirez-Ramirez et al., 2015; Peterson, et al. 2003). Previous studies observed similar results that diets supplemented with long chain UFA or triglyceride decreased yields of de novo-synthesized and mixed-source FA, but increased yield of preformed FA (Ramirez-Ramirez, et al., 2016; Boerman and Lock, 2014). Therefore, the supplementation of long chain FA in MFDI might have compensated the reduction of preformed FA yield.

Due to variation in feed ingredients and nutrient composition in the studies described above (Bradford and Allen, 2004; Rico et al., 2014), it is possible that differing mechanisms caused the response to MFD by higher and lower producing cows. Although it was not determined in the study, Bradford and Allen (2004) assumed that lower producing cows were less able to maintain stable and high rumen pH, and therefore experienced greater negative impact on BH pathways in the rumen and milk fat synthesis in the mammary gland. Rico et al. (2014) speculated that the higher rumen passage rate of higher producing cows resulted in more MFD-associated BH intermediates escaping from the rumen and decreased milk fat synthesis in the mammary gland. Both studies observed higher MFD-associated BH intermediates in milk from cows with more pronounced MFD. In our experiment, MFDI increased the concentration of *t*10, *c*12 CLA in milk compared with CON, and higher producing cows showed a greater increase in the concentration of this FA than lower producing cows. Additionally, *t*10, *c*12 CLA in milk was highly correlated with milk fat concentration. The BH theory of MFD explains that MFD is caused by inhibition of milk fat synthesis in the mammary gland by specific

intermediates formed during BH of unsaturated FA (Bauman et al., 2011). Both *t*10, *c*12 CLA and *t*9, *c*11 CLA are intermediates formed during rumen BH of unsaturated FA, which have been shown to inhibit milk fat synthesis (Baumgard et al., 2001; Perfield et al., 2007). *T*10, *c*12 CLA is the most well studied. There is a curvilinear relationship between increasing abomasal infusions of *t*10, *c*12 CLA and the extent of reduction in milk fat synthesis (Shingfield and Griinari, 2007). McClymont and Vallance (1962) previously proposed that the elevated insulin associated with MFD diets results in prioritization of energy and nutrient utilization toward body tissues other than the mammary gland. Although plasma insulin was increased by MFDI in our study, we did not observe a correlation between milk fat concentration and insulin concentration. It is believed that insulin plays a more important role in regulating energy balance during MFD than causing MFD (Bauman and Griinari, 2001). Plasma insulin is negatively correlated with yield of milk fat; however, this may be due to the fact that insulin is also negatively correlated with milk yield.

MFD is often associated with alterations in energy partitioning (Harvatine et al., 2009; Fernandes et al., 2014; Boerman et al., 2015b). A meta-analysis by Harvatine et al. (2009) showed that *t*10, *c*12 CLA-induced MFD decreased milk energy output by 16% compared with control treatments. Fernandes et al. (2014) observed an increase in energy balance of dairy goats treated with increasing doses of a *t*10, *c*12 CLA methyl esters supplement. Boerman et al. (2015b) reported that a high-starch diet decreased milk energy output and increased energy retained in body tissue compared with a high-fiber diet supplemented with fat. However, we did not observe changes in calculated energy balance or energy partitioning in our study. Harvatine et al. (2009) reported that *t*10, *c*12 CLA-induced MFD up-regulated expression of enzymes and key regulators of lipid synthesis in dairy cows and speculated that adipose tissue storage was a

result of spared energy from reduced milk fat synthesis. Although MFDI up-regulated expressions of lipogenic enzymes, including *ACACA*, *LIPE*, and *ELOVL6* (all $P < 0.10$; 28, 25 and 35%, respectively), in our study (data not published), it did not affect BW, BCS, or thickness of rump and rib fat (data not published). MFDI decreased milk fat yield, but increased yields of milk, milk protein, and milk lactose. Therefore, treatment had no effect on milk energy output.

Dietary source of PUFA and alterations in the rumen environment and bacteria population are considered requirements for diet-induced MFD (Bauman et al., 2011). Dietary unsaturated FA are toxic to rumen bacteria and BH reduces toxicity (Maia et al., 2007 and 2010). Many FA intermediates are produced during the BH of 18 carbon FA, and *c*9, *t*11 CLA and *t*11 18:1 represent two predominant isomers (Bauman and Griinari, 2001). However, risk factors for MFD can cause a shift in the BH pathway for *c*9, *c*12 18:2 and increase formation of other intermediates, including *t*10, *c*12 CLA and *t*10 18:1 (Bauman and Griinari, 2001). As mentioned above, *t*10, *c*12 CLA is one of the potent inhibitors of milk fat synthesis, and *t*10 18:1 is often used as a marker for MFD despite its lack of effect on milk fat synthesis (Lock et al., 2007). By using an in vitro batch culture system, we previously studied the effects of different risk factors for MFD, including culture pH, oil concentration, starch content, and starch fermentability, on BH pathways of *c*9, *c*12 18:2 (Sun et al., 2014 and 2015). Among all tested factors, culture pH was shown to have the greatest impact on BH, and increasing corn oil concentration or starch content and fermentability at low pH resulted in greater accumulation of *t*10, *c*12 CLA at low culture pH than at high culture pH (Sun et al., 2014 and 2015).

In our current study, MFDI decreased mean rumen pH and minimum rumen pH and increased pH range. This reduction in rumen pH was expected and is typically observed in cows fed a high-concentrate diet (Bauman and Griinari, 2001). Rumen bacteria are sensitive to pH

changes, especially the cellulolytic bacteria including *Ruminococcus albus*, *Bacteroides succinogenes*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens* (Russell and Dombrowski, 1980). Cellulolytic bacteria play important roles in BH pathways. *Butyrivibrio spp.* isomerize *c*9, *c*12 18:2 to *c*9, *t*11 CLA and *t*9, *t*12 18:2 and hydrogenate those intermediates to form *trans* FA (McKain et al., 2010). However, *Megasphaera elsdenii*, a producer of *t*10, *c*12 CLA (Kim, et al., 2002), is more tolerant of low pH, and still grows in cultures until pH reaches 4.9 (Russell and Dombrowski, 1980). Therefore, a reduction in rumen pH inhibits the growth and metabolism of *Butyrivibrio spp.*, decreases the BH extent of *c*9, *c*12 18:2, and increases formation of *t*10, *c*12 CLA (Trogegeler-Meynadier et al., 2006; Fuentes et al., 2011; Sun et al., 2014 and 2015). We also observed that, with increasing preliminary milk yield, pH range was consistent in CON fed cows, but was increased in MFDI fed cows. Both high dietary starch and fat supplementation have been shown to increase rumen pH range (Oba and Allen, 2003; Rico et al., 2014). Greater pH fluctuations in higher producing cows might have had a greater impact on rumen bacteria population and BH pathways than in lower producing cows. Additionally, rumen pH range is highly positively correlated with *t*10, *c*12 CLA in both milk and rumen, which indicates that greater rumen pH range likely increases formation of *t*10, *c*12 CLA in rumen.

In our current study, the CON increased intake of *c*9, *c*12, *c*15 18:3 compared with MFDI, especially in the higher producing cows. This might have been caused by the greater DMI of CON by higher producing cows and the fact that CON contained more *c*9, *c*12, *c*15 18:3-enriched forages than MFDI. *C*9, *c*12, *c*15 18:3 accounted for less than 10% of the total FA. Compared with CON, MFDI increased RUFAL and intakes of the other two major unsaturated FA, *c*9 18:1 and *c*9, *c*12 18:2. Our previous in vitro batch culture study showed that increasing corn oil at low culture pH resulted in greater increases in *t*10, *c*12 CLA and *t*10 18:1 than at high

culture pH (Sun et al., 2014). Increased substrates for BH and low rumen pH might have combined to increase formation of *t*10, *c*12 CLA in the rumen of MFDI fed cows. Our individual rumen FA pool size results are consistent with this. MFDI increased the rumen pools of *t*10, *c*12 CLA and *t*10 18:1 by 24 and 58%, respectively. A Pearson correlation analysis showed that the rumen pool of *t*10, *c*12 CLA was highly positively correlated with milk *t*10, *c*12 CLA concentration and highly negatively associated with milk fat concentration. Shingfield and Griinari (2007) reported that abomasal infusion of *t*10, *c*12 CLA decreased milk fat concentration and yield, and that there was a curvilinear reduction in milk fat synthesis with increasing *t*10, *c*12 CLA dose. Omasal or duodenal flow of *t*10, *c*12 CLA ranged from 0.30 to 1.40 g/d, depending on fat supplementation, when cows were fed a high-concentrate diet (Shingfield and Griinari, 2007). We did not measure the passage rate and absorption of *t*10, *c*12 CLA in our study, and the rumen pool size was not sufficient to represent production and passage rate of this FA. However, it has been proposed that high DMI in high producing cows may increase the rumen passage rate of nutrients and lead to more *t*10, *c*12 CLA passing from the rumen and being absorbed in the intestines (Rico et al., 2014). We also observed effects of treatment diets on rumen VFA molar proportions. Most notably, MFDI increased propionate compared with CON, and the increase was greater in higher producing cows than lower producing cows. Furthermore, Pearson correlation analysis showed that milk fat concentration was negatively correlated with propionate molar proportion. Maxin et al. (2011) infused *t*10, *c*12 CLA, propionate, and acetate in dairy cows, and found that propionate had an additive effect on *t*10, *c*12 CLA-induced MFD. Besides inhibition of *t*10, *c*12 CLA on milk fat synthesis, increased propionate may have also contributed to the greater MFD observed in higher producing cows fed MFDI.

Boerman et al. (2015ab) reported that diets containing high levels of starch decreased NDF total tract digestibility. Studies have reported both decreases and increases in total tract NDF digestibility as a result of diets supplemented with long chain FA (Hristov et al., 2005; Piantoni et al., 2013 and 2015b; Sun et al., 2014). In our current study, MFDI contained higher starch and was supplemented with FA, and decreased total tract NDF digestibility in comparison to CON. The reduction in NDF total tract digestibility of cows fed MFDI could have been caused by a number of factors including 1) a reduction in rumen pH and cellulolytic bacteria in the rumen (Russell and Wilson, 1996; Calsamiglia et al., 2002; Sun et al., 2014 and 2015), 2) an increased passage rate of NDF from the rumen (Oba and Allen, 2003c) and 3) high soybean hulls content in CON, which is more digestible (Boerman et al., 2015). Although turnover rate of NDF was decreased by MFDI, it is unknown if rumen passage rate was decreased because NDF digestion in the rumen was not measured. Ueda et al. (2003) found that a high concentrate (starch) diet reduced total tract digestibility, but did not affect NDF digestibility in the rumen, which might have been due to compensatory NDF digestion in the large intestine. Piantoni et al. (2013 and 2015a) found that diets supplemented with palmitic acid or stearic acid resulted in decreased total tract FA digestibility. We also found that MFDI diet increased total FA intake and rumen pool, but decreased total FA total tract digestibility compared to CON. The decreased FA digestibility might have been caused by insufficient emulsification or lipolysis of triglyceride associated with increasing dietary fat supplementation, as suggested by Palmquist (1991).

CONCLUSIONS

MFDI, contained low NDF, high starch and total FA, tended to induce MFD compared with CON. Higher producing cows exhibited greater reductions in both milk fat yield and concentration when fed MFDI, compared with CON. This interaction between treatment and

production level interaction was likely due to the effect of rumen pH changes on BH pathways, which led to increased formation of intermediates associated with MFD (*e.g.* *t*10, *c*12 CLA). Future research should focus on the effects of diet on fractional rates of rumen FA BH and passage. This would provide further information that could clarify the relationship between production level and MFD risk in dairy cows.

APPENDIX

APPENDIX

Table 5.1. Ingredients and nutrient composition of treatment diets[1].

	Treatments	
	CON	MFDI
Ingredient, % of DM		
Corn silage[2]	35.5	29.9
Alfalfa silage[3]	13.5	8.46
Ground corn	10.2	10.1
High moisture corn	6.09	17.8
Soybean meal	15.9	16.9
Wheat straw	1.69	1.69
Soybean hulls	9.30	3.38
Cottonseed	4.57	7.61
Ca-salt palm FA[4]	-	0.85
Vitamin & mineral mix[5]	3.23	3.23
Nutrient composition		
DM, %	50.6	54.7
NDF, % of DM	33.7	28.1
Forage NDF, % of DM	21.2	19.0
Starch, % of DM	22.7	28.9
CP, % of DM	16.9	16.9
Total FA, % of DM	2.91	4.27

[1]Average composition of experimental diets fed to 32 cows in a crossover design with 28-d treatment periods. Values are based on nutrient composition of individual ingredients sampled during the last 5 d of each period. Treatments were either control (CON, control diet; with 33.7% NDF, 21.2% forage NDF, 22.7% starch, and 2.91% total FA on a DM basis) or milk fat depression-inducing diet (MFDI, milk fat depression-inducing diet; with 28.1% NDF, 19.0% forage NDF, 28.9% starch, and 4.27% total FA on a DM basis).
[2] Corn silage, 42.6% NDF and 9.85% iNDF.
[3]Alfalfa silage, 35.2% NDF and 13.2% iNFD.
[4]Megalac, Arm & Hammer, Princeton, NJ
[5]Vitamin and mineral mix contained 20.6% dry ground shell corn, 21.5 sodium bicarbonate, 18.1% limestone, 15.5% white salt, 13.2% calcium carbonate, 5.5% Biofos (The Mosaic Co., Plymouth, MN), 2.4% magnesium oxide, 1.2% soybean oil, and < 1% of each of the following:

Table 5.1. (cont'd)
Sodium bicarbonate, manganese sulfate, zinc sulfate, ferrous sulfate, copper sulfate, iodine, cobalt carbonate, vitamin E, vitamin A, vitamin D, and selenium.

Table 5.2. Dry matter intake, milk production, milk components, and feed efficiency for cows fed treatment diets (n = 30) [1].

	Trt[2]		SEM	*P*-value [3]	
	CON	MFDI		Trt	Trt × PMY
DMI, kg	26.4	26.5	0.49	0.80	0.004
Milk yield, kg/d					
Milk	36.3	37.6	0.52	0.01	0.29
3.5% FCM [4]	37.0	35.6	0.97	0.06	0.05
ECM [5]	37.0	36.3	0.89	0.23	0.13
Milk components					
Fat, kg/d	1.28	1.21	0.05	0.05	0.06
Fat, %	3.69	3.31	0.12	<0.001	0.01
Protein, kg/d	1.15	1.20	0.02	<0.001	0.01
Protein, %	3.22	3.24	0.04	0.44	0.18
Lactose, kg/d	1.74	1.81	0.03	0.01	0.18
Lactose, %	4.78	4.80	0.02	0.16	0.64
BW[6]	743	746	11	0.08	0.97
BCS[6]	3.35	3.40	0.05	0.12	0.92
Change in BW (kg/d)	0.68	0.84	0.09	0.30	0.95
Change in BCS (pt/28 d)	0.11	0.18	0.02	0.02	0.57
ECM/DMI	1.36	1.35	0.03	0.56	0.49

[1] Samples and data for production variables collected from d 22 to 26 of each treatment period.
[2] Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.
[3] *P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).
[4] Fat-corrected milk; 3.5% FCM = [(0.4324 × kg of milk) + (16.216 × kg of milk fat)].
[5] Energy-corrected milk; ECM = [(0.327 × kg of milk) + (12.95 × kg of milk fat) + (7.20 × kg of milk protein)].
[6] Mean throughout the 28-d period.

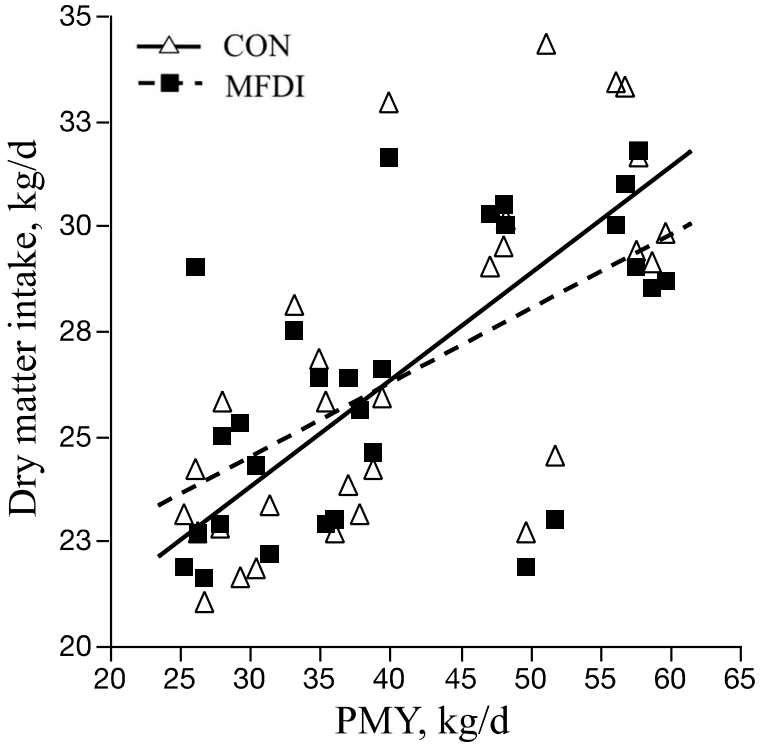

Figure 5.1. Relationship between DMI and preliminary milk yield of cows fed either CON or MFDI.

Relationship between DMI and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; DMI [kg/d] = 16.2 + 0.254 × PMY [kg/d]; R^2 = 0.54; $P < 0.01$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; DMI [kg/d] = 19.2 + 0.176 × PMY [kg/d]; $R^2 = 0.37$; $P < 0.01$; dashed line and square markers). PMY = preliminary milk yield. $P = 0.80$ for treatment effect; $P = 0.004$ for interaction between treatment and preliminary milk yield.

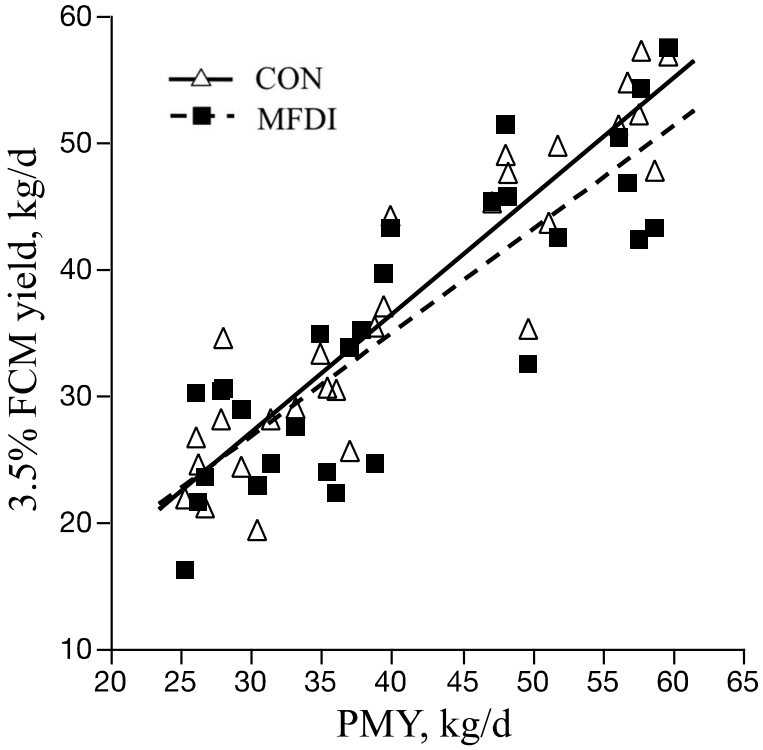

Figure 5.2. Relationship between 3.5% FCM yield and preliminary milk yield of cows fed either CON or MFDI.

Relationship between 3.5% FCM yield and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; 3.5% FCM yield [kg/d] = -0.878 + 0.932 × PMY [kg/d]; $R^2 = 0.86$; $P < 0.01$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet low forage and high concentrate diet; n = 30; 3.5% FCM yield [kg/d] = 2.27 + 0.817 × PMY [kg/d]; $R^2 = 0.73$; $P < 0.01$; dashed line and square markers). PMY = preliminary milk yield. $P = 0.06$ for treatment effect; $P = 0.05$ for interaction between treatment and preliminary milk yield.

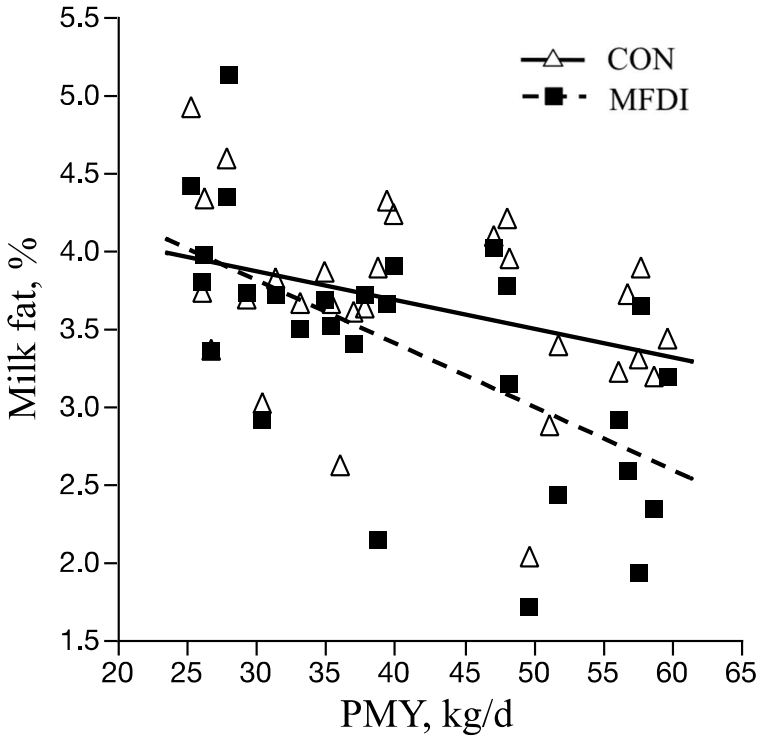

Figure 5.3. Relationship between milk fat percentage and preliminary milk yield of cows fed either CON or MFDI.

Relationship between milk fat percentage and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; milk fat [%] = 4.42 – 0.018 × PMY [kg/d]; $R^2 = 0.13$; $P = 0.06$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; milk fat [%] = 5.03 – 0.041 × PMY [kg/d]; $R^2 = 0.37$; $P < 0.01$; dashed line and square markers). PMY = preliminary milk yield. $P < 0.001$ for treatment effect; $P = 0.01$ for interaction between treatment and preliminary milk yield.

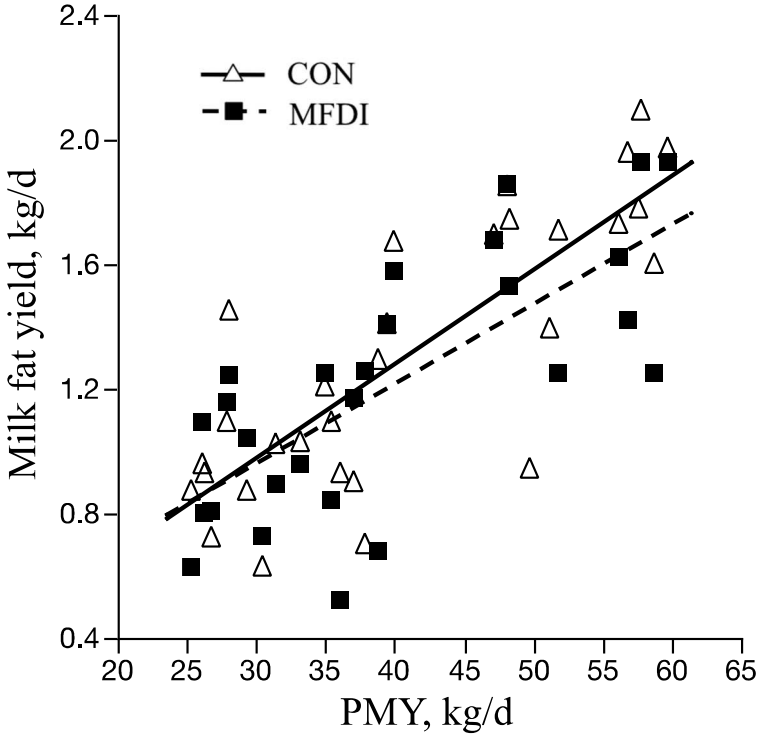

Figure 5.4. Relationship between milk fat yield and preliminary milk yield of cows fed either CON or MFDI.

Relationship between milk fat yield and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; milk fat yield [kg/d] = 0.072 + 0.030 × PMY [kg/d]; $R^2 = 0.65$; $P < 0.01$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; milk fat yield [kg/d] = 0.193 + 0.026 × PMY [kg/d]; $R^2 = 0.53$; $P < 0.01$; dashed line and square markers). PMY = preliminary milk yield. $P = 0.05$ for treatment effect; $P = 0.06$ for interaction between treatment and preliminary milk yield.

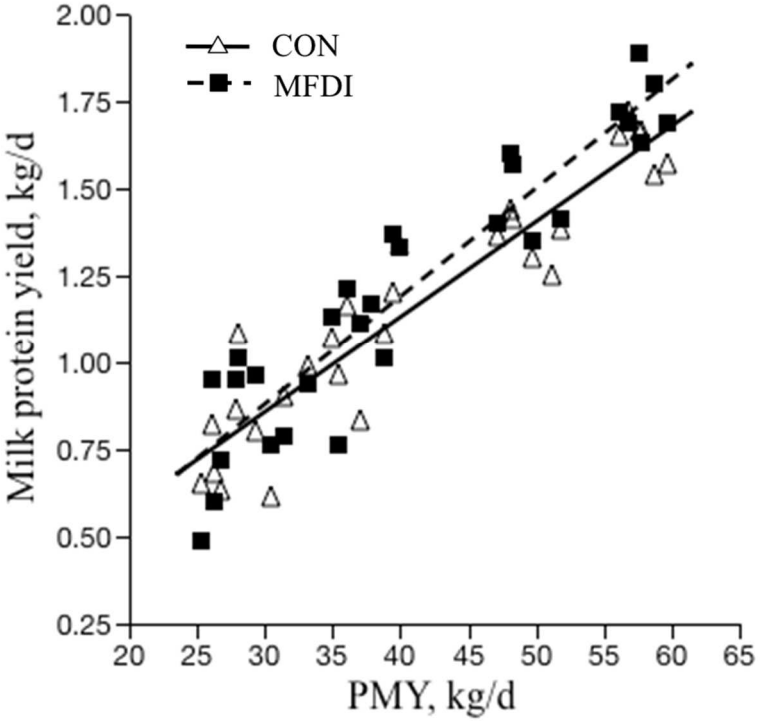

Figure 5.5. Relationship between milk protein yield and preliminary milk yield of cows fed either CON or MFDI.

Relationship between milk protein yield and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; milk protein yield [kg/d] = -0.066 + 0.029 × PMY [kg/d]; $R^2 = 0.84$; $P < 0.001$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; milk protein yield [kg/d] = 0.041 + 0.029 × PMY [kg/d]; $R^2 = 0.89$; $P < 0.001$; dashed line and square markers). PMY = preliminary milk yield. $P < 0.001$ for treatment effect; $P = 0.01$ for interaction between treatment and preliminary milk yield.

Table 5.3. FA intake and RUFAL of cows fed treatment diets (n=30)[1].

Item, g/d	Trt[2]			*P*-value[3]	
	CON	MFDI	SEM	Trt	Trt × PMY
16:0	129	243	3.68	<0.001	0.10
18:0	20	31	0.49	<0.001	0.49
*c*9 18:1	133	249	3.78	<0.001	0.12
*c*9, *c*12 18:2	380	511	8.39	<0.001	0.84
*c*9, *c*12, *c*15 18:3	46	36	0.76	<0.001	<0.001
RUFAL[4]	560	796	12.8	<0.001	0.85
Total FA	769	1129	18.0	<0.001	0.70

[1]Based on animal performance throughout the 28-d treatment periods unless otherwise stated.
[2]Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.
[3]*P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).
[4]Summation of *c*9 18:1, *c*9, *c*12 18:2, and *c*9, *c*12, *c*15 18:3.

Table 5.4. Milk FA concentration of cows fed treatment diets (n=30).

Item, g/100 g	Trt[1]		SEM	*P*-value[2]	
	CON	MFDI		Trt	Trt × PMY
Selected individual FA[3]					
4:0	3.32	3.01	0.09	0.001	0.01
6:0	2.27	1.93	0.07	<0.001	0.006
8:0	1.38	1.15	0.04	<0.001	0.01
10:0	3.39	2.81	0.11	<0.001	0.04
12:0	3.94	3.29	0.11	<0.001	0.15
iso 13:0	0.03	0.03	< 0.01	<0.001	0.04
aiso 13:0	0.08	0.06	< 0.01	<0.001	0.74
13:0	0.20	0.18	0.01	0.02	0.27
iso 14:0	0.10	0.07	< 0.01	<0.001	0.97
14:0	11.8	10.4	0.17	<0.001	0.97
iso 15:0	0.28	0.20	< 0.01	<0.001	0.21
aiso 15:0	0.48	0.39	0.01	<0.001	0.62
*c*9 14:1	0.92	0.89	0.05	0.31	0.01
15:0	1.03	0.95	0.02	0.01	0.003
16:0	29.9	28.9	0.35	<0.001	0.06
*c*9 16:1	1.57	1.69	0.09	0.15	0.05
17:0	0.62	0.53	0.01	<0.001	0.01
18:0	10.3	10.2	0.27	0.71	0.008
*t*4 18:1	0.02	0.03	< 0.01	<0.001	0.29
*t*5 18:1	0.02	0.02	< 0.01	<0.001	0.05
*t*6 + *t*7 + *t*8 18:1	0.30	0.48	0.02	<0.001	0.05
*t*9 18:1	0.23	0.34	0.01	<0.001	0.16
***t*10 18:1**	**0.60**	**2.12**	**0.37**	**0.002**	**0.19**
***t*11 18:1**	**0.98**	**1.35**	**0.07**	**0.001**	**0.30**
*t*12 18:1	0.48	0.64	0.02	<0.001	0.34

Table 5.4. (cont'd)

Item, g/100 g	Trt[1]		SEM	*P*-value[2]	
	CON	MFDI		Trt	Trt × PMY
*c*9 18:1	17.6	19.1	0.29	<0.001	0.60
*c*11 18:1	0.46	0.63	0.02	<0.001	0.002
*c*12 18:1	0.54	0.70	0.02	<0.001	0.32
*c*13 18:1	0.09	0.14	0.01	<0.001	0.01
*c*14 + *t*16 18:1	0.37	0.37	0.01	0.83	0.10
*c*9, *c*12 18:2	2.72	3.28	0.07	<0.001	0.18
*c*9, *t*11 CLA	0.45	0.63	0.04	0.001	0.14
_t_9, _c_11 CLA	**0.01**	**0.02**	**< 0.01**	**0.001**	**0.16**
_t_10, _c_12 CLA	**< 0.01**	**0.01**	**< 0.01**	**<0.001**	**0.07**
*c*9, *c*12, *c*15 18:3	0.41	0.32	0.01	<0.001	0.01
Unknown	3.22	3.12	0.05	0.005	<0.01
Summation of FA[4]					
De Novo	**27.0**	**23.5**	**0.51**	**<0.001**	**0.13**
Mixed	**31.4**	**30.6**	**0.34**	**0.001**	**0.39**
Preformed	**41.6**	**45.9**	**0.65**	**<0.001**	**0.02**
OBCFA	2.82	2.42	0.04	<0.001	0.001

[1] Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.
[2] *P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).
[3]A total of approximately 70 individual FA were quantified and used for calculations (summation by concentrations). Only selected FA are reported in the table.
[4] De novo milk FA originate from mammary gland de novo synthesis (< 16 carbons in length); preformed milk FA originate from mobilized FA or dietary FA (> 16 carbons in length); mixed, milk FA originate from both sources (16-carbons in length); OBCFA, odd- and branched- chain FA, summation of *iso* 13:0, *aiso* 13:0, 13:0, *iso* 14:0, *iso* 15:0, 15:0, 17:0

Table 5.5. Milk fatty acid yield of cows fed treatment diets (n=30).

Item, g/d	Trt[1]		SEM	*P*-value[2]	
	CON	MFDI		Trt	Trt × PMY
Select individual FA[3]					
4:0	41.1	34.7	2.26	<0.001	<0.001
6:0	28.2	22.4	1.65	<0.001	<0.001
8:0	17.1	13.3	1.02	<0.001	<0.001
10:0	42.0	32.4	2.48	<0.001	0.001
12:0	48.4	37.6	2.68	<0.001	0.002
iso 13:0	0.41	0.29	0.02	<0.001	0.02
aiso 13:0	0.93	0.72	0.05	<0.001	0.01
13:0	2.40	2.09	0.14	0.007	0.19
iso 14:0	1.21	0.73	0.06	<0.001	<0.001
14:0	144	118	6.58	<0.001	0.002
iso 15:0	3.32	2.22	0.12	<0.001	<0.001
aiso 15:0	5.76	4.36	0.20	<0.001	0.006
*c*9 14:1	11.1	9.77	0.54	0.001	0.96
15:0	12.6	10.8	0.58	<0.001	0.48
16:0	369	326	17.5	<0.001	<0.001
*c*9 16:1	19.1	18.2	0.74	0.06	0.87
17:0	7.43	5.92	0.27	<0.001	0.008
18:0	121	112	5.86	0.04	0.004
*t*4 18:1	0.22	0.29	0.01	<0.001	0.22
*t*5 18:1	0.18	0.26	0.01	<0.001	0.04
*t*6 + *t*7 + *t*8 18:1	3.51	5.19	0.13	<0.001	<0.001
*t*9 18:1	2.67	3.69	0.10	<0.001	0.01
_t_10 18:1	**7.14**	**19.6**	**2.22**	**<0.001**	**0.006**
_t_11 18:1	**11.7**	**15.5**	**1.03**	**0.01**	**0.18**
*t*12 18:1	5.61	7.12	0.29	<0.001	0.51

Table 5.5. (cont'd)

Item, g/d	Trt[1]		SEM	*P*-value[2]	
	CON	MFDI		Trt	Trt × PMY
*c*9 18:1	209	209	7.54	0.97	0.11
*c*11 18:1	5.51	6.82	0.21	<0.001	0.008
*c*12 18:1	6.37	7.80	0.35	0.002	0.66
*c*13 18:1	1.00	1.45	0.06	<0.001	<0.001
*c*14 + *t*16 18:1	4.35	4.18	0.24	0.48	0.04
*c*9, *c*12 18:2	32.4	35.7	1.10	0.005	0.60
*c*9, *t*11 CLA	5.34	7.15	0.46	0.01	0.11
_t_9, _c_11 CLA	**0.06**	**0.22**	**0.03**	**<0.001**	**0.002**
_t_10, _c_12 CLA	**0.02**	**0.11**	**0.02**	**<0.001**	**0.01**
*c*9, *c*12, *c*15 18:3	4.85	3.48	0.13	<0.001	<0.001
Unknown	38.3	34.3	1.17	0.002	0.21
Summation of FA[4]					
De Novo	**332**	**268**	**16.5**	**<0.001**	**0.001**
Mixed	**388**	**344**	**17.8**	**<0.001**	**<0.001**
Preformed	**493**	**501**	**16.3**	**0.64**	**0.20**
OBCFA	34.0	27.1	1.32	<0.001	0.02

[1] Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.
[2] *P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).
[3] A total of approximately 70 individual FA were quantified and used for calculations (summation by concentrations). Only selected FA are reported in the table.
[4] De novo milk FA originate from mammary gland de novo synthesis (< 16 carbons in length); preformed milk FA originate from mobilized FA or dietary FA (> 16 carbons in length); mixed, milk FA originate from both sources (16:0 + *c*9 16:1); OBCFA, odd- and branched- chain FA, summation of *iso* 13:0, *aiso* 13:0, 13:0, *iso* 14:0, *iso* 15:0, 15:0, 17:0.

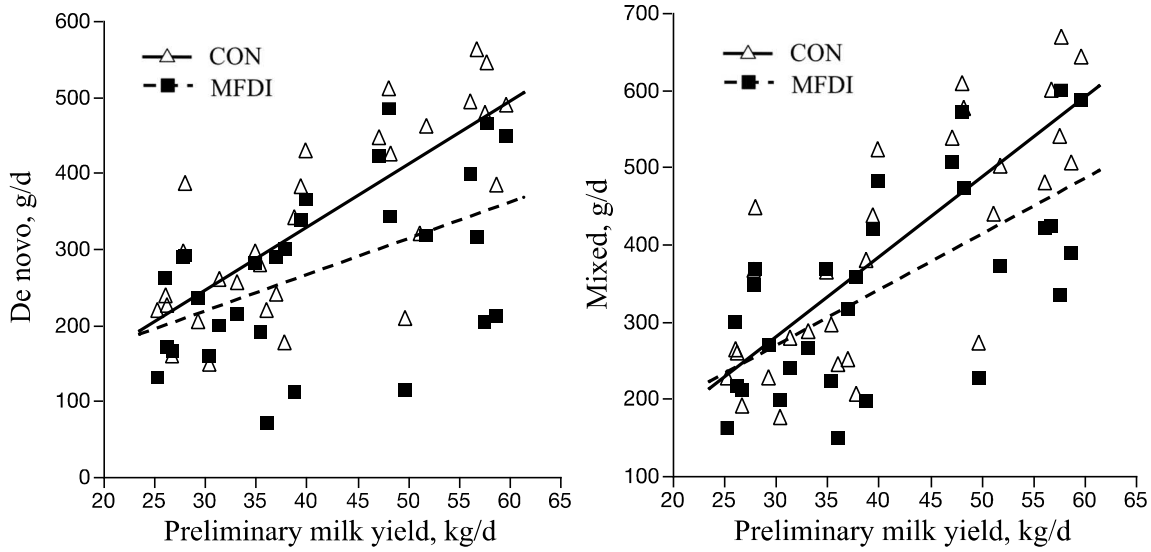

Figure 5.6. Relationship between yields of de novo milk FA and mixed FA and preliminary milk yield of cows fed either CON or MFDI.
Relationship between de novo milk FA yield (left) and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; de novo milk FA yield [g/d] = -3.30 + 8.28 × PMY [kg/d]; $R^2 = 0.60$; $P < 0.01$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; de novo milk FA yield [g/d] = 75.4 + 4.76 × PMY [kg/d]; $R^2 = 0.25$; $P < 0.01$; dashed line and square markers); relationship between mixed milk FA yield (right) and preliminary milk yield of cows fed either CON (high forage and low concentrate diet; n = 30; mixed milk FA yield [g/d] = -31.2 + 10.4 × PMY [kg/d]; $R^2 = 0.63$; $P < 0.01$; solid line and triangle markers) or MFDI (low forage and high concentrate diet; n = 30; mixed milk FA yield [g/d] = 52.1 + 7.22 × PMY [kg/d]; $R^2 = 0.43$; $P < 0.01$; dashed line and square markers); PMY = preliminary milk yield. Both $P < 0.001$ for treatment effect; both $P \leq 0.001$ for interaction between treatment and preliminary milk yield.

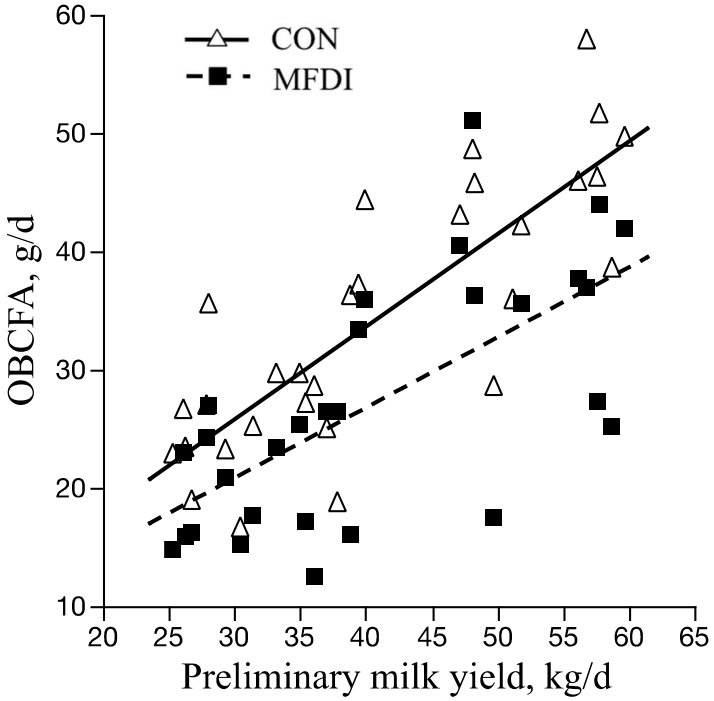

Figure 5.7. Relationship between odd- and branched- chain FA yield and preliminary milk yield of cows fed either CON or MFDI.

Relationship between odd- and branched- chain FA yield and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; OBCFA [g/d] = 2.30 + 0.783 × PMY [kg/d]; $R^2 = 0.67$; $P < 0.01$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; OBCFA [g/d] = 3.02 + 0.594 × PMY [kg/d]; $R^2 = 0.45$; $P < 0.01$; dashed line and square markers). OBCFA = odd- and branched- chain FA; PMY = preliminary milk yield. $P < 0.001$ for treatment effect; $P = 0.02$ for interaction between treatment and preliminary milk yield.

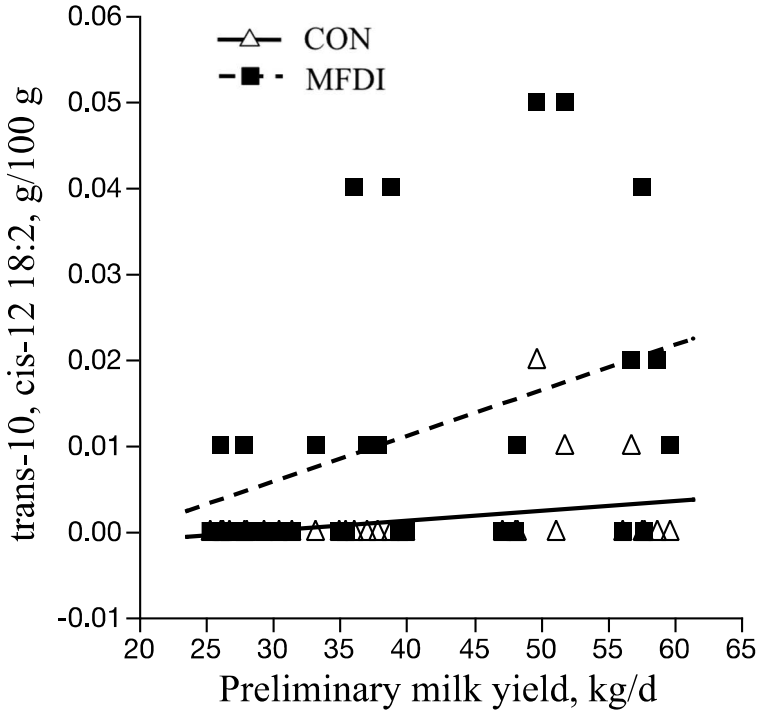

Figure 5.8. Relationship between *t*10, *c*12 CLA concentration and preliminary milk yield of cows fed either CON or MFDI.

Relationship between *t*10, *c*12 CLA concentration and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; *t*10, *c*12 CLA [g/100 g] = -0.003 + 0.00011 × PMY [kg/d]; $R^2 = 0.09$; $P = 0.10$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; *t*10, *c*12 CLA [g/100 g] = -0.010 + 0.00053 × PMY [kg/d]; $R^2 = 0.14$; $P = 0.05$; dashed line and square markers). PMY = preliminary milk yield. $P < 0.001$ for treatment effect; $P = 0.07$ for interaction between treatment and preliminary milk yield.

Table 5.6. Body weight, BCS and calculated energy values for cows fed treatment diets (n=30)[1].

Variable	Trt[2]		SEM	*P*-value[3]	
	CON	MFDI		Trt	Trt × PMY
Calculated energy values					
Apparent NE_L of diet[4] (Mcal/kg)	1.55	1.59	0.03	0.36	0.69
Milk (Mcal/d)	25.2	25.0	0.67	0.68	0.16
Body energy gain (Mcal/d)	4.39	5.50	0.62	0.30	0.84
Maintenance (Mcal/d)	11.4	11.4	0.13	0.10	0.95
Partitioning (% energy intake)					
Milk	60.2	58.7	1.22	0.44	0.91
Body tissue gain	11.0	13.4	1.39	0.33	0.95
Maintenance	28.8	28.1	0.73	0.41	0.46

[1]Based on animal performance throughout the 28-d periods unless otherwise stated.
[2]Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.
[3]*P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).
[4]From sum of milk energy output, maintenance energy calculated from metabolic BW, and body energy gain divided by DMI for each cow on each diet throughout the 28-d period.

Table 5.7. Plasma concentrations of glucose and insulin of cows fed experimental diets (n=30).

Item	Trt		SEM	*P*-value	
	CON	MFD		Trt	Trt × PMY
Plasma Glucose, mg/dL	62.5	63.6	0.60	0.06	<0.001
Plasma Insulin, ug/L	1.32	1.61	0.08	<0.001	0.96

[1] Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.
[2] *P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY)

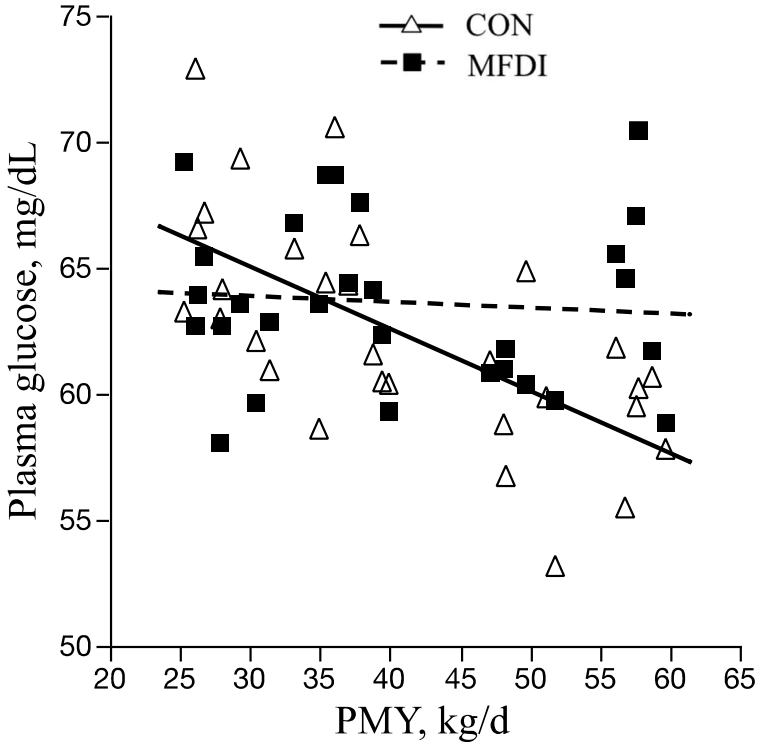

Figure 5.9. Relationship between glucose and preliminary milk yield of cows fed either CON or MFDI.

Relationship between glucose and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 30; plasma glucose [mg/dL] = 72.4 – 0.246 × PMY [kg/d]; $R^2 = 0.42$; $P < 0.01$; solid line and etriangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 30; plasma glucose [mg/dL] = 64.6 – 0.023 × PMY [kg/d]; $R^2 = 0.01$; $P = 0.68$; dashed line and square markers). PMY = preliminary milk yield. $P = 0.06$ for treatment effect; $P < 0.001$ for interaction between treatment and preliminary milk yield.

Table 5.8. Rumen pH of rumen-cannulated cows fed treatment diets (n = 13).

Item	Trt[1]		SEM	*P*-value[2]	
	CON	MFDI		Trt	Trt × PMY
Mean pH	6.13	6.00	0.03	<0.001	0.05
Maximum pH	6.73	6.78	0.05	0.36	0.01
Minimum pH	5.65	5.49	0.04	0.001	0.97
pH range[3]	1.09	1.29	0.06	0.01	0.03

[1]Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA. Rumen fluid samples were collected every 15 h over 5 d, resulting in 8 samples per cow per period that represented every 3 h of a 24-h period to account for diurnal variation.
[2]*P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).
[3]pH range is calculated as the difference between maximum pH and minimum pH.

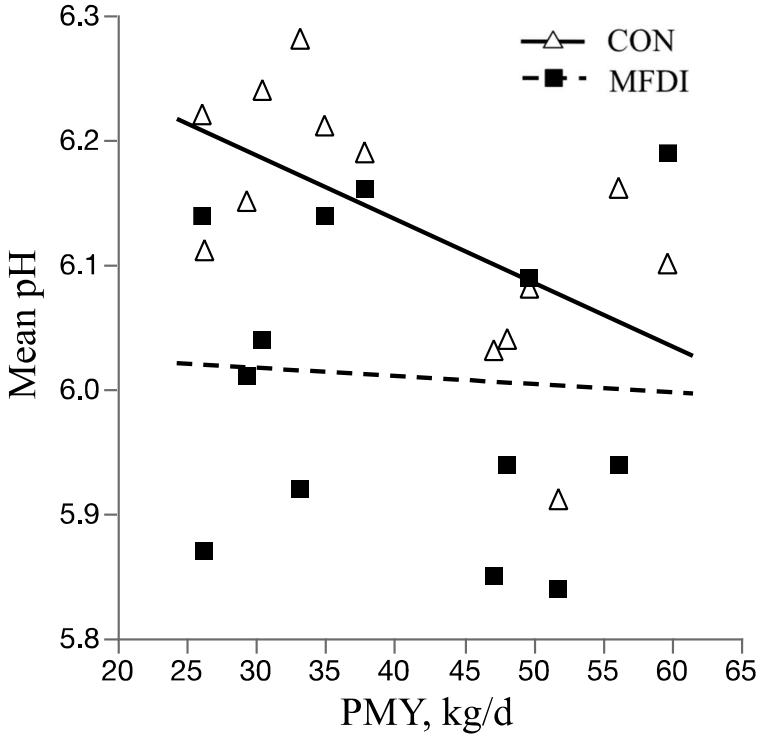

Figure 5.10. Relationship between mean rumen pH and preliminary milk yield of cows fed either CON or MFDI.

Relationship between mean rumen pH and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 13; mean pH = 6.341 - 0.005 × PMY [kg/d]; $R^2 = 0.35$; $P = 0.03$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 13; mean pH = 6.037 - 0.001 × PMY [kg/d]; $R^2 = 0.004$; $P = 0.84$; dashed line and square markers). PMY = preliminary milk yield. $P < 0.001$ for treatment effect; $P = 0.05$ for interaction between treatment and preliminary milk yield.

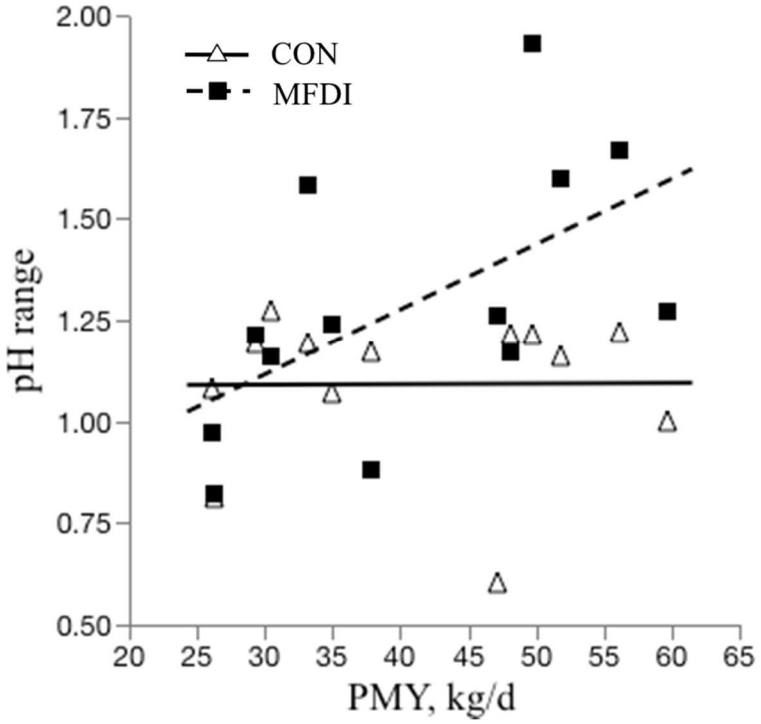

Figure 5.11. Relationship between rumen pH range and preliminary milk yield of cows fed either CON or MFDI.

Relationship between rumen pH range and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 13; pH range = 1.085 + 0.0001 × PMY [kg/d]; $R^2 < 0.001$; $P = 0.98$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 13; pH range = 0.632 + 0.016 × PMY [kg/d]; $R^2 = 0.34$; $P = 0.04$; dashed line and square markers). PMY = preliminary milk yield. $P = 0.01$ for treatment effect; $P = 0.03$ for interaction between treatment and preliminary milk yield.

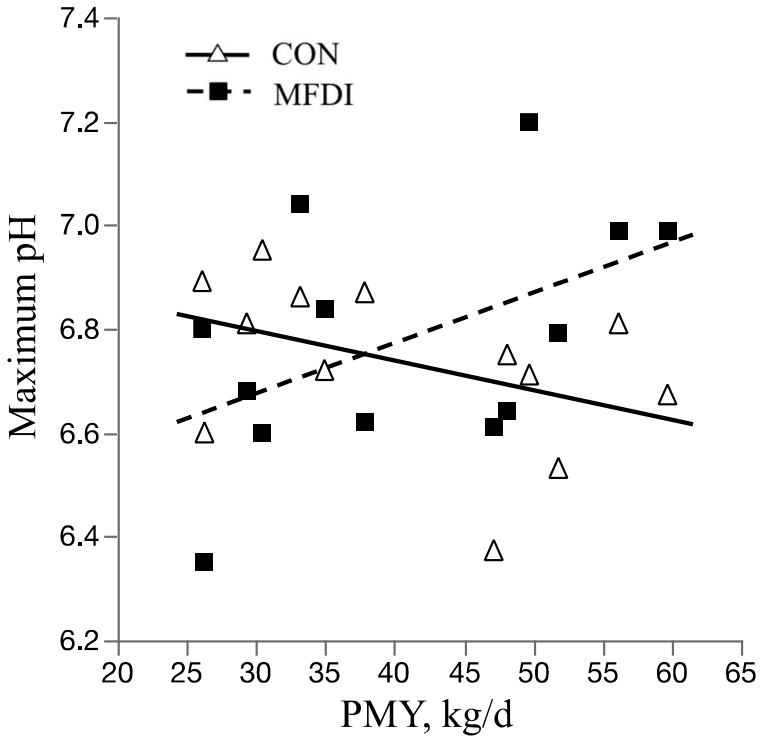

Figure 5.12. Relationship between maximum rumen pH and preliminary milk yield of cows fed either CON or MDFI.

Relationship between maximum rumen pH and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 13; maximum pH = 6.966 - 0.006 × PMY [kg/d]; $R^2 = 0.17$; $P = 0.16$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 13; maximum pH = 6.385 + 0.010 × PMY [kg/d]; $R^2 = 0.24$; $P = 0.09$; dashed line and square markers). PMY = preliminary milk yield. $P = 0.36$ for treatment effect; $P = 0.01$ for interaction between treatment and preliminary milk yield.

Table 5.9. Rumen VFA concentrations of rumen-cannulated cows fed treatment diets (n = 13).

Item	Trt[1]		SEM	*P*-value[2]	
	CON	MFDI		Trt	Trt × PMY
Total VFA, mM	107	105	4.18	0.64	0.83
Individual VFA, mol/100 mol					
Acetate (A)	62.0	57.6	0.55	<0.001	0.99
Propionate (P)	21.1	24.5	0.66	<0.001	0.06
Isobutyrate	0.82	0.88	0.04	0.38	0.18
Butyrate	12.8	13.5	0.38	0.09	0.004
Isovalerate	1.44	1.34	0.08	0.07	0.17
Valerate	1.79	2.09	0.17	<0.001	0.17
A:P	2.98	2.40	0.09	<0.001	0.24

[1]Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON) or a low forage, high concentrate diet (MFDI) supplemented with 1% Ca-salt palm FA.
[2]*P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).

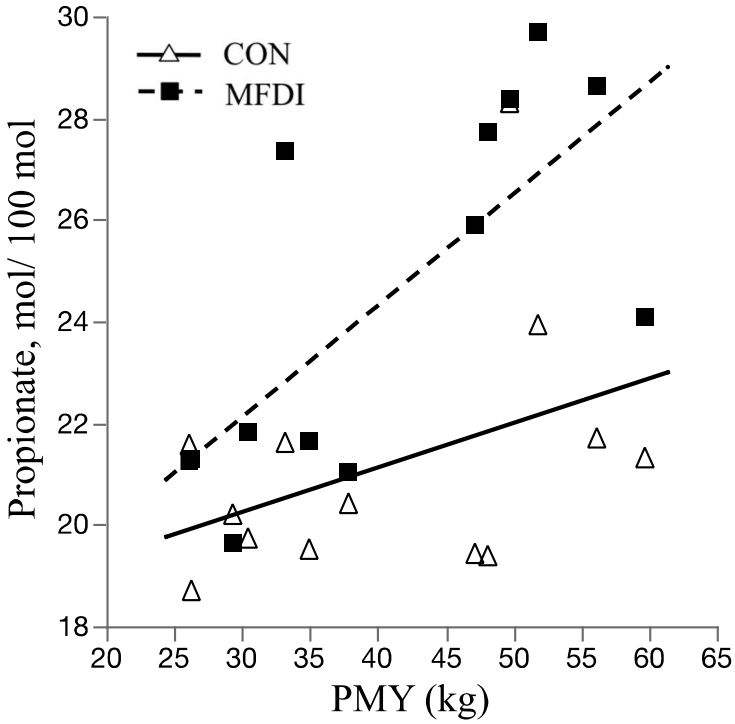

Figure 5.13. Relationship between molar proportion of propionate and preliminary milk yield of cows fed either CON or MFDI.

Relationship between molar proportion of propionate and preliminary milk yield of cows fed either CON (control, high forage and low concentrate diet; n = 13; molar proportion of propionate = 17.6 + 0.088 × PMY [kg/d]; $R^2 = 0.16$; $P = 0.17$; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 13; molar proportion of propionate = 15.5 + 0.219 × PMY [kg/d]; $R^2 = 0.53$; $P = 0.005$; dashed line and square markers). PMY = preliminary milk yield. $P < 0.001$ for treatment effect; $P = 0.06$ for interaction between treatment and preliminary milk yield.

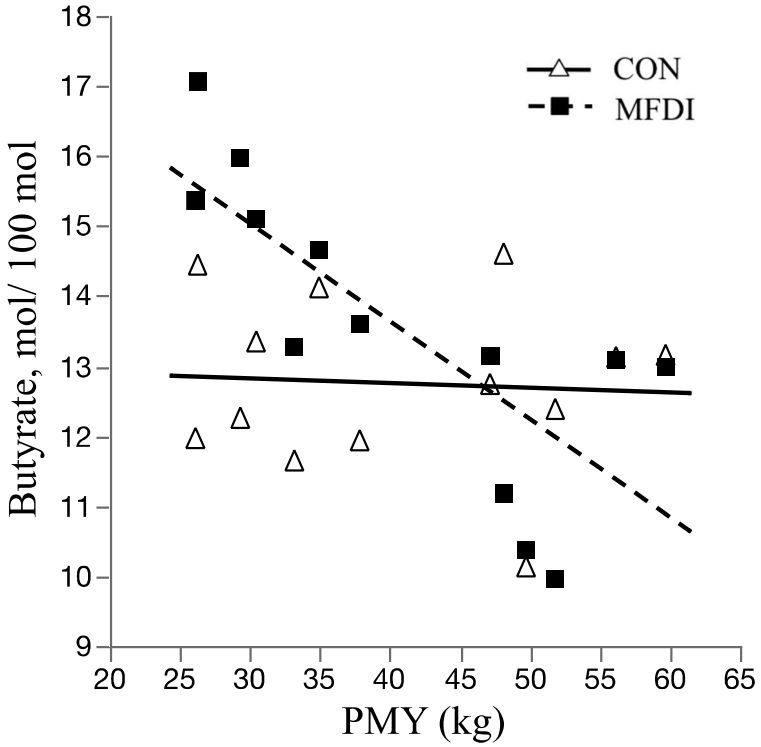

Figure 5.14. Relationship between molar proportion of butyrate and preliminary milk yield of cows fed either CON or MFDI.

Relationship between molar proportion of butyrate and preliminary milk yield of cows fed either CON (control diet, high forage and low concentrate diet; n = 13; molar proportion of butyrate [mol/100 mol] = 13.0 - 0.007 × PMY [kg/d]; R_2 = 0.004; *P* = 0.84; solid line and triangle markers) or MFDI (milk fat depression-inducing diet, low forage and high concentrate diet; n = 13; molar proportion of butyrate [mol/100 mol] = 19.2 - 0.140 × PMY [kg/d]; R_2 = 0.60; *P* = 0.002; dashed line and square markers). PMY = preliminary milk yield. *P* = 0.09 for treatment effect; *P* = 0.004 for interaction between treatment and preliminary milk yield.

Table 5.10. Nutrient digestibilities, intakes, rumen pool sizes, and turnover rates of rumen-cannulated cows fed treatment diets (n = 13).

Item	Trt[1]		SEM	*P*-value[2]	
	CON	MFDI		Trt	Trt × PMY
Total tract digestibility, %					
DM	65.2	64.1	0.65	0.14	0.21
NDF	42.7	31.2	1.09	<0.001	0.11
Total FA	81.7	76.4	1.32	0.005	0.47
Apparent NE_L of diet[3] (Mcal/kg)	1.61	1.61	0.01	0.94	0.20
Nutrient intake, kg/d					
NDF	8.74	7.50	0.27	<0.001	0.002
iNDF	2.28	2.32	0.07	0.31	0.003
Total FA	0.77	1.15	0.03	<0.001	0.03
Nutrient rumen pool, kg					
Wet matter	82.0	78.1	3.27	0.04	0.51
DM	12.8	12.5	0.75	0.59	0.80
NDF	7.26	7.04	0.46	0.30	0.72
iNDF	2.78	2.71	0.16	0.44	0.76
Total FA	0.53	0.66	0.04	<0.001	0.39
Rumen mass volume, L	97.3	93.4	3.69	0.17	0.85
Nutrient turnover rate, %/h					
NDF	5.28	4.74	0.34	0.02	0.12
iNDF	3.60	3.74	0.24	0.35	0.13
Total FA	6.37	7.65	0.49	0.002	0.08

[1]Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.
[2]*P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).
[3]From digestibility equations (NRC, 2001) based on nutrient digestibility results collected during the last 5 d of each treatment period.

Table 5.11. Rumen FA pool size of rumen-cannulated cows fed treatment diets (n = 13)[1].

Item, g	Trt[2]		*P*-value[3]	
	CON	MFDI	Trt	Trt × PMY
Selected individual FA				
12:0	0.80	0.87	0.08	0.23
13:0	0.35	0.31	0.04	0.69
iso 14:0	0.94	0.79	<0.001	0.49
14:0	3.72	4.76	<0.001	0.33
iso 15:0	2.23	1.86	0.003	0.27
aiso 15:0	3.72	3.62	0.18	0.73
*c*9 14:1	5.68	4.72	0.002	0.34
15:0	3.10	2.66	0.001	0.79
iso 16:0	1.06	0.84	0.002	0.01
16:0	94.9	137	<0.001	0.67
*c*7 + *c*8 16:1	0.73	0.64	0.01	0.90
*c*9 16:1	0.92	1.22	0.001	0.60
*c*10 + *t*13 16:1	0.99	0.87	0.04	0.16
17:0	1.67	1.65	0.59	0.69
18:0	158	177	0.04	0.12
*t*6 + *t* 7 + *t*8 18:1	2.09	3.15	<0.001	0.99
*t*9 18:1	1.43	2.04	<0.001	0.92
*t*10 18:1	4.79	7.56	<0.001	0.62
*t*11 18:1	11.3	13.4	0.10	0.76
*t*12 18:1	3.40	4.43	<0.001	0.46
*c*9 18:1	46.2	70.1	<0.001	0.81
*c*11 18:1	6.19	7.78	<0.001	0.47
*c*12 18:1	4.71	5.21	0.25	0.71
*c*13 18:1	0.24	0.29	0.01	0.88
*c*14 and *t*16 18:1	2.90	2.63	0.63	0.26
19:0	0.63	0.70	0.02	0.20

Table 5.11. (cont'd)

Item, g	Trt[2]		*P*-value[3]	
	CON	MFDI	Trt	Trt × PMY
*c*9, *c*12 18:2	97.7	124	0.01	0.38
*c*6, *c*9, *c*12 18:3	2.51	2.77	0.02	0.81
*c*9, *c*12, *c*15 18:3	7.53	6.00	0.02	0.19
*c*11 20:1	0.51	0.63	0.004	0.19
*c*9, *t*11 CLA	1.88	1.80	0.85	0.62
*t*10, *c*12 CLA	0.45	0.56	0.04	0.59
*c*11, *c*14 20:2	0.11	0.12	0.26	0.93
*c*8, *c*11, *c*14 20:3	1.82	1.78	0.54	0.79
23:0	0.61	0.54	0.01	0.46
Unknown	21.5	18.3	0.001	0.79
Summation of FA[4]				
SFA	265	328	<0.001	0.16
PUFA	110	135	0.01	0.37
CLA	2.46	3.09	<0.001	0.13
OBCFA	13.2	12.0	<0.001	0.33

[1] To meet the assumption of homogeneity of variance, data were transformed (reciprocal) before analysis. For the purpose of interpretation, means were back-transformed and included in the table. 95% confidence intervals of the values were back-transformed and presented in Supplement Table 1.

[2]Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression inducing diet) supplemented with 1% Ca-salt palm FA.

[3]*P*-value associated with treatment differences (CON vs. MFDI; Trt) and the linear interaction between treatment and preliminary milk yield (Trt × pMY).

[4]SFA, saturated FA, summation of 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 23:0; PUFA, poly-unsaturated FA, summation of *c*9, *c*12 18:2, *c*6, *c*9, *c*12 18:3, *c*9, *c*12, *c*15 18:3, *c*11, *c*14 20:2, *c*8, *c*11, *c*14 20:3; CLA, conjugated linoleic acids, summation of *c*9, *t*11 CLA and *t*10, *c*12 CLA; OBCFA, odd- and branched- chain FA, summation of 13:0, *iso* 14:0, *iso* 15:0, *aiso* 15:0, 15:0, *iso* 16:0, 17:0.

Table 5.12. Correlation coefficients among production variables, plasma insulin and metabolites, and milk FA for cows fed treatment diets (n = 30).

Item	Milk Yield, kg	Milk Fat Yield, kg/d	Milk Fat, %	BCS Change	Plasma Glucose, mg/dL	Plasma Insulin, ug/L	Milk *t*10, *c*12 CLA, g/100 g	Milk *t*10 18:1, g/100 g
Milk Yield	1	0.77[1]	-0.44	-0.27	-0.42	-0.61	0.30	0.17
		(<.001)[2]	(<.001)	(0.04)	(<.001)	(<0.001)	(0.02)	(0.19)
Milk Fat Yield		1	0.21	-0.18	-0.55	-0.63	-0.22	-0.33
			(0.11)	(0.18)	(<0.001)	(<0.001)	(0.09)	(0.01)
Milk Fat %			1	0.16	-0.10	0.01	-0.69	-0.71
				(0.22)	(0.45)	(0.95)	(<0.001)	(<0.001)
BCS Change				1	0.25	0.18	-0.14	-0.02
					(0.06)	(0.17)	(0.30)	(0.86)
Plasma Glucose					1	0.60	0.00	0.14
						(<0.001)	(1.00)	(0.30)
Plasma Insulin						1	-0.02	0.10
							(0.87)	(0.46)
Milk *t*10, *c*12 CLA							1	0.88
								(<0.001)
Milk *t*10 18:1								1

[1]The Pearson correlation coefficient of the linear relationship between 2 variables.
[2]The *P*-value associated with the linear relationship between 2 variables.

Table 5.13. Correlation coefficients among production variables, milk FA concentration, rumen FA pool, and rumen pH of rumen-cannulated cows fed treatment diets (n = 13).

Item	Milk fat yield, kg	Milk fat, %	Milk *t*10, *c*12 CLA, g/100 g	Milk *t*9, *c*11 CLA g/100 g	Rumen *t*10, *c*12 CLA, g	*c*9, *c*12 18:2 intake, g/d	Total RUFAL, g/d	Rumen mean pH	Rumen max pH	Rumen min pH	Rumen pH range	Propionate, mol/100 mol
Milk fat yield	1	0.21[1]	-0.15	-0.23	-0.04	0.48	0.43	-0.26	-0.20	-0.08	-0.09	0.15
		(0.30)[2]	(0.46)	(0.25)	(0.86)	(0.01)	(0.03)	(0.21)	(0.33)	(0.70)	(0.66)	0.48
Milk fat %		1	-0.74	-0.80	-0.66	0.08	0.05	0.05	-0.50	0.54	-0.69	-0.66
			(<0.001)	(<0.001)	(<0.001)	(0.71)	(0.81)	(0.82)	(0.009)	(0.004)	(<0.001)	(<0.001)
Milk *t*10, *c*12 CLA			1	0.84	0.94	-0.02	0.02	-0.24	0.36	-0.53	0.59	0.64
				(<0.001)	(<0.001)	(0.93)	(0.93)	(0.23)	(0.07)	(0.005)	(0.002)	(<0.001)
Milk *t*9, *c*11 CLA				1	0.72	-0.03	0.01	-0.11	0.45	-0.49	0.63	0.63
					(<0.001)	(0.89)	(0.97)	(0.60)	(0.02)	(0.01)	(<0.001)	(<0.001)
Rumen *t*10, *c*12 CLA					1	-0.03	0.00	-0.35	0.26	-0.66	0.60	0.64
						(0.90)	(0.99)	(0.08)	(0.19)	(<0.001)	(0.001)	(<0.001)
*c*9, *c*12 18:2 intake						1	1.00	-0.44	0.08	-0.29	0.24	0.42
							(<0.001)	(0.03)	(0.71)	(0.15)	(0.24)	(0.03)
Total RUFAL							1	-0.44	0.08	-0.31	0.25	0.44
								(0.02)	(0.68)	(0.12)	(0.21)	(0.03)
Rumen mean pH								1	0.36	0.73	-0.20	-0.54
									(0.07)	(<0.001)	(0.32)	(0.004)
Rumen maximum pH									1	-0.13	0.78	0.36
										(0.52)	(<0.001)	(0.07)
Rumen minimum pH										1	-0.72	-0.74
											(<0.001)	(<0.001)
Rumen pH range											1	0.71
												(<0.001)
Propionate												1

[1]The Pearson correlation coefficient of the linear relationship between 2 variables.
[2]The *P*-value associated with the linear relationship between 2 variables

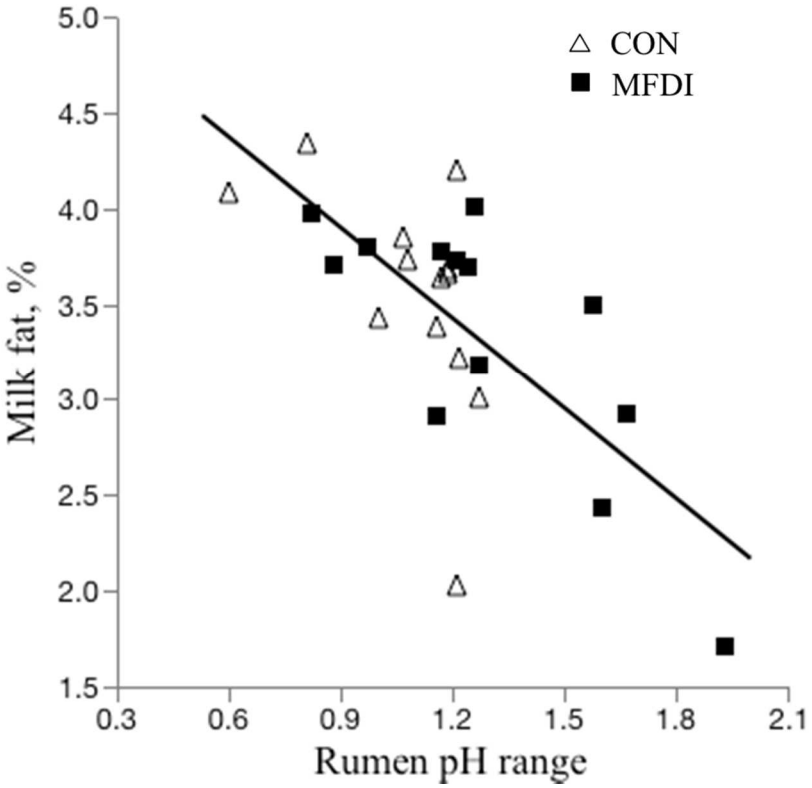

Figure 5.15. Relationship between milk fat concentration and rumen pH range of cows fed either CON or MFDI.

Relationship between milk fat concentration and rumen pH range of cows fed either CON (control diet) or MFDI (milk fat depression-inducing diet; milk fat concentration [%] = 5.32 – 1.574 × rumen pH range; $R_2 = 0.48$; $P < 0.001$). CON contained high forage and low concentrate (n = 13; triangle markers). MFDI contained low forage and high concentrate diet (n = 13; square markers).

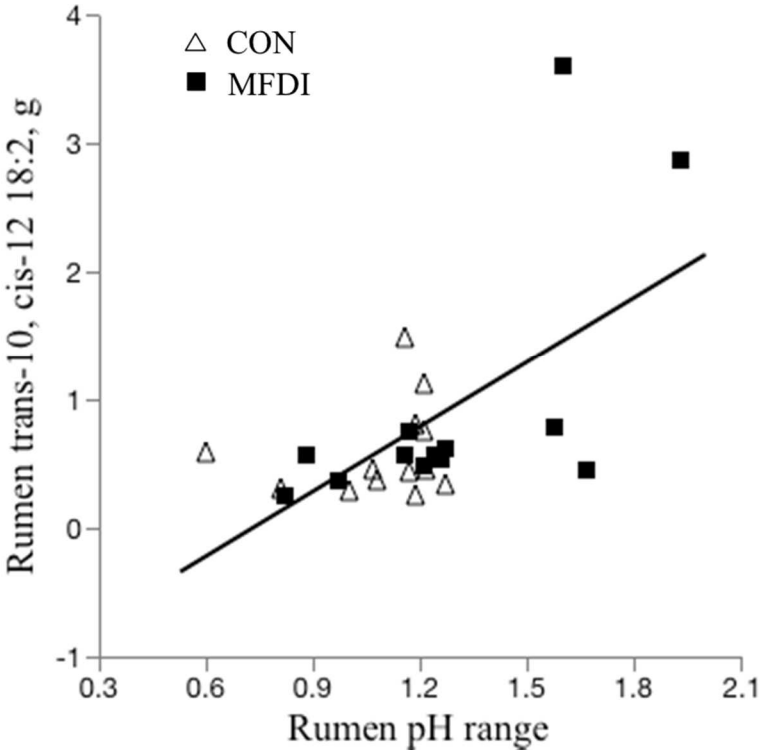

Figure 5.16. Relationship between rumen *t*10, *c*12 CLA and rumen pH range of cows fed either CON or MFDI.

Relationship between rumen *t*10, *c*12 CLA and rumen pH range of cows fed either CON (control diet) or MFDI (milk fat depression-inducing diet; rumen *t*10, *c*12 CLA [g] = - 1.23 + 1.679 × rumen pH range; $R_2 = 0.36$; $P = 0.001$). CON contained high forage and low concentrate (n = 13; triangle markers). MFDI contained low forage and high concentrate diet (n = 13; square markers).

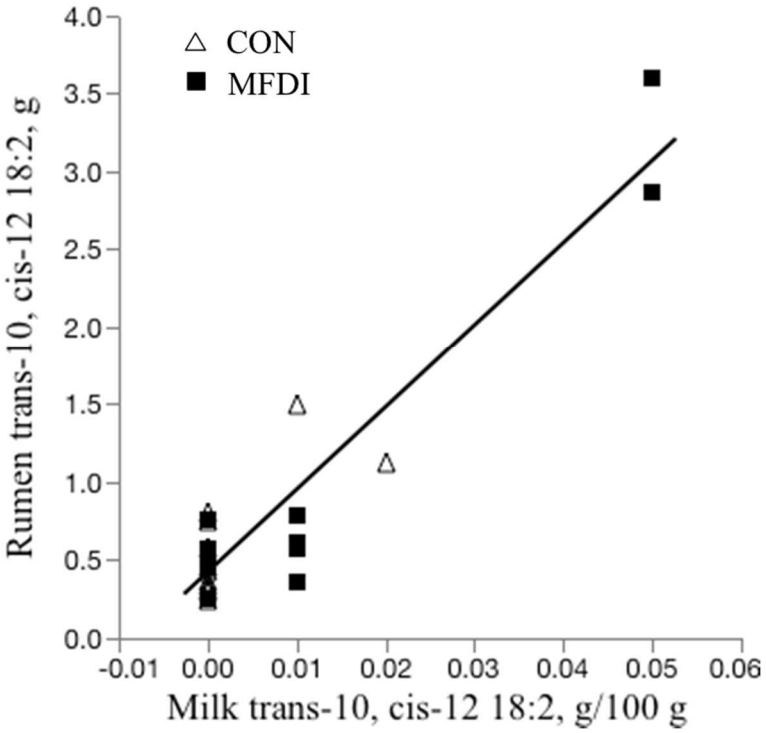

Figure 5.17. Relationship between rumen *t*10, *c*12 CLA and milk *t*10, *c*12 CLA of cows fed either CON or MFDI.

Relationship between rumen *t*10, *c*12 CLA and milk *t*10, *c*12 CLA of cows fed either CON (control diet) or MFDI (milk fat depression-inducing diet; rumen *t*10, *c*12 CLA [g] = 0.424 + 52.9 × milk *t*10, *c*12 CLA [g/100 g]; R_2 = 0.0.87; $P < 0.001$). CON contained high forage and low concentrate (n = 13; triangle markers). MFDI contained low forage and high concentrate diet (n = 13; square markers).

Supplemental Table 1. 95% confidence intervals of the rumen FA pool size of rumen-cannulated cows fed treatment diets[1].

Item, g/100 g	CON[2]		MFDI	
	Lower 95% CI	Upper 95% CI	Lower 95% CI	Upper 95% CI
Selected individual FA				
12:0	0.7	0.94	0.75	1.03
13:0	0.3	0.41	0.28	0.36
iso 14:0	0.86	1.04	0.73	0.86
14:0	3.3	4.27	4.09	5.7
iso 15:0	2.06	2.44	1.74	2
aiso 15:0	3.34	4.2	3.26	4.07
*c*9 14:1	5.12	6.37	4.33	5.19
15:0	2.8	3.48	2.44	2.94
iso 16:0	0.92	1.25	0.75	0.96
16:0	85.9	106	119	162
*c*7 + *c*8 16:1	0.65	0.83	0.58	0.72
*c*9 16:1	0.79	1.1	1.01	1.56
*c*10 + *t*13 16:1	0.89	1.12	0.79	0.96
17:0	1.49	1.91	1.47	1.88
18:0	143	177	158	200
*t*6 + *t 7* + *t*8 18:1	1.89	2.33	2.72	3.73
*t*9 18:1	1.3	1.58	1.79	2.36
*t*10 18:1	4.06	5.85	5.89	10.6
*t*11 18:1	10.1	12.8	11.7	15.7
*t*12 18:1	3.11	3.74	3.96	5.03
*c*9 18:1	41.1	52.8	58.9	86.4
*c*11 18:1	5.62	6.9	6.89	8.93
*c*12 18:1	4.16	5.45	4.54	6.12
*c*13 18:1	0.21	0.27	0.25	0.35
*c*14 and *t*16 18:1	2.23	4.15	2.06	3.62

Supplemental Table 1 (cont'd)

Item, g/100 g	CON[2]		MFDI	
	Lower 95% CI	Upper 95% CI	Lower 95% CI	Upper 95% CI
19:0	0.55	0.73	0.61	0.83
*c*9, *c*12 18:2	84.1	117	103	156
*c*6, *c*9, *c*12 18:3	2.25	2.83	2.46	3.16
*c*9, *c*12, *c*15 18:3	6.29	9.4	5.18	7.12
*c*11 20:1	0.45	0.6	0.53	0.77
*c*9, *t*11 CLA	1.38	2.99	1.33	2.79
*t*10, *c*12 CLA	0.36	0.59	0.43	0.79
*c*11, *c*14 20:2	0.1	0.13	0.1	0.15
*c*8, *c*11, *c*14 20:3	1.62	2.07	1.59	2.01
23:0	0.54	0.69	0.49	0.61
Unknown	19.7	23.7	17	19.9
Summation of FA				
∑ SFA	240	294	292	374
∑ PUFA	94.8	131	113	168
∑ CLA	2.21	2.77	2.7	3.6
∑ OBCFA	12.1	14.6	11.1	13.1

[1]95% confidence intervals of the values were back-transformed.
[2]Trt = dietary treatments. Treatments consisted of either a high forage and low concentrate diet (CON, control diet) or a low forage, high concentrate diet (MFDI, milk fat depression-inducing diet) supplemented with 1% Ca-salt palm FA.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The studies described in this dissertation determined the effects of dietary factors and rumen pH on biohydrogenation (BH) pathways and formation of BH intermediates associated with milk fat depression (MFD; *t*10, *c*12 conjugated linoleic acid [CLA]) by using both in vitro and in vivo methodologies. Of the tested factors in the in vitro studies (Chapter 2-4), culture pH had the greatest impact on BH pathways. Unsaturated FA content, starch content, starch fermentability, and *Saccharomyces cerevisiae* fermentation product interacted with culture pH to influence formation of *t*10, *c*12 CLA in vitro. Low pH inhibited both the isomerisation and hydrogenation steps of BH and caused a shift from the *t*11 pathway to the *t*10 pathway. Additionally, increasing unsaturated FA, the substrate for BH, at low pH resulted in greater formation of *t*10, *c*12 CLA than at high pH. High starch content, combined with a highly fermentable starch source (high moisture corn), increased formation of *t*10, *c*12 CLA at low pH and this effect was partially mediated through pH reduction during incubation. *Saccharomyces cerevisiae* fermentation product decreased formation of *t*10, *c*12 CLA in cultures containing highly fermentable starch at low pH. This may have been mediated through changes in bacterial growth and metabolism. Future research focused on the interactions between dietary factors and rumen pH and their effects on rumen bacterial population and metabolism is needed.

In support of our in vitro results, diets containing high FA (provided by Ca-salt palm FA and cottonseed) and high starch content (provided by high moisture corn) successfully induced MFD (Chapter 5). In contrast to our hypothesis, higher producing cows fed this MFD-inducing diet exhibited greater reductions in milk fat yield and content and increases in milk *t*10, *c*12 CLA content than did lower producing cows. In consistent with our in vitro studies, rumen pH

exhibited greater fluctuation in higher producing cows, which experienced greater MFD. Rumen pH range is highly negatively correlated with milk fat concentration, and highly positively correlated with concentration of *t*10, *c*12 CLA in milk and *t*10, *c*12 CLA pool in the rumen. Although the rumen pool of *t*10, *c*12 CLA was increased by the MFD-inducing diet, we did not observe an interaction between production level and diet for this variable. The formation of *t*10, *c*12 CLA in the rumen and its passage rate to the intestine are not clear. Future investigations should focus on the effects of MFD-inducing diets on fractional rates of BH and passage of FA from the rumen, and whether the results are consistent across different types of MFD-inducing diets. Additionally, due to conflicting results from previous studies that tested the impact of production level on diet-induced MFD, future research on the relationship between production level and diet-induced MFD is needed. Future results will help researchers understand the mechanisms behind MFD and determine whether they are consistent across diets. Moreover, this information will help nutritionists alleviate MFD on dairy farms and develop effective feeding strategies targeted to maximize milk fat yield throughout lactation.

Overall, rumen pH had the greatest impact on BH pathways and milk fat synthesis of the factors tested in our studies, and pH reduction was the major driver of increased formation of *t*10, *c*12 CLA and decreased milk fat synthesis. These results allow us to develop effective dietary strategies to alleviate MFD and maximize milk fat yield on dairy farm via maintaining a stable and high rumen pH.