Effect of diet fermentability and unsaturated fatty acid concentration on recovery from diet-induced milk fat depression

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ABSTRACT

Diet-induced milk fat depression is caused by highly fermentable and high-unsaturated fatty acid (FA) diets, and results in reduced milk fat concentration and yield, reduced de novo FA, and increased *trans* isomers of the alternate biohydrogenation pathways. The hypothesis of the current experiment was that a diet higher in fermentability and lower in unsaturated FA (UFA) would accelerate recovery compared with a high-UFA and lower-fermentability diet. Eight ruminally cannulated and 9 noncannulated multiparous Holstein cows were randomly assigned to treatment sequences in a replicated Latin square design. During each period milk fat depression was induced for 10 d by feeding a low-fiber, high-UFA diet [25.9% neutral detergent fiber (NDF) and 3.3% C18:2]. Following the induction phase, cows were switched to recovery treatments for 18 d designed to correct dietary fermentability, UFA, or both fermentability and UFA concentration. Treatments during recovery were (1) correction of fiber and UFA diet [control; 31.8% NDF and 1.65% C18:2], (2) a diet predominantly correcting fiber, but not UFA [high oil (HO); 31.3% NDF and 2.99% C18:2], and (3) a diet predominantly correcting UFA, but not fiber concentration [low fiber (LF); 28.4% NDF and 1.71% C18:2]. Milk and milk component yield, milk FA profile, ruminal pH, and 11 rumen microbial taxa were measured every third day during recovery. Milk yield decreased progressively in HO and control, whereas it was maintained in the LF diet. Milk fat concentration increased progressively during recovery in all treatments, but was on average 9% lower in LF than control from d 12 to 18. Milk fat yield increased progressively in all treatments and was not different between control and LF at any time

point, but was lower in HO than control on d 15. Milk *trans*-10 C18:1 and *trans*-10,*cis*-12 conjugated linoleic acid decreased progressively in all treatments, but was higher in HO than control from d 3 to 18 [136 ± 50 and $188 \pm 57\%$ (mean $\pm$ SD)], whereas LF caused a smaller increase in these FA compared with control (67 ± 25 and $90 \pm 22\%$). Additionally, milk *trans*-11 C18:1 and *cis*-9,*trans*-11 conjugated linoleic acid was decreased in control and LF and increased in HO during recovery. Selected microbial species observed changed during recovery, but major treatment differences were only observed for *Streptococcus bovis*. The LF diet that was similar in UFA but 3.4% units lower in NDF compared with to the control had a similar decrease in alternate *trans* biohydrogenation intermediates in milk. The HO diet that was similar in NDF but 2.0% units higher in UFA compared with the control had higher alternate *trans* biohydrogenation intermediates in milk compared with control. However, recovery of milk fat yield was similar between treatments at most time points.

Key words: milk fat depression, dairy cow, conjugated linoleic acid

INTRODUCTION

Milk fat is the most variable milk component on farms and is especially responsive to nutritional factors. Classical diet-induced milk fat depression (MFD) is a specific reduction in milk fat concentration and yield commonly observed when dairy cows are fed highly fermentable and high-unsaturated fatty acid (UFA) diets (Bauman and Griinari, 2001). Specific *trans* FA, such as *trans*-10,*cis*-12 CLA, formed as intermediates during altered ruminal biohydrogenation of linoleic acid have been identified as the causative factors of diet-induced MFD (Harvatine et al., 2009). The alternate biohydrogenation (BH) pathway results from a change in rumen environment, altered rumen microbial population, or a large amount of UFA requiring BH (Lourenço et al., 2010; Weimer et al., 2010). Diet-induced MFD has been extensively studied (reviewed by Harvatine et al., 2009); however, recovery from MFD and mechanisms to accelerate recovery have only recently been investigated

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Table 1. Example treatment sequences in the 3×3 Latin square design investigating the effect of dietary fiber and unsaturated fatty acid (UFA) concentration on recovery from milk fat depression¹

Treatment sequence	Period 1, 10/18 d	Washout, 7 d	Period 2, 10/18 d	Washout, 7 d	Period 3, 10/18 d
1	MFD/Control	Control	MFD/LF	Control	MFD/HO
2	MFD/HO	Control	MFD/Control	Control	MFD/LF
3	MFD/LF	Control	MFD/HO	Control	MFD/Control

¹Milk fat depression (MFD) was achieved by feeding the induction diet (low fiber, high UFA) during the first 10 d of each period. Treatments were a high fiber, low UFA diet (control), a diet correcting UFA, but not fiber level (Low fiber: LF), and a diet correcting fiber, but not UFA (high oil: HO) fed for 18 d during the recovery phase. Monensin was fed in all diets.

(Rico and Harvatine, 2013; Rico et al., 2014a,b). The current experiment uses a similar experimental model to investigate recovery from MFD.

Dietary starch, fiber, and UFA concentration, rate of fermentation, and monensin are some of the risk factors for MFD. Monensin is commonly fed to dairy cows to increase feed efficiency; however, it has been associated with lower milk fat concentration concomitantly with increased milk *trans* FA, including *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA, and reduced concentration of de novo synthesized FA in milk (Duffield et al., 2008). In addition, monensin supplementation in high-UFA diets increased the concentration of intermediates of the alternate BH pathway in rumen fluid and milk (AlZahal et al., 2008; He et al., 2012). The current experiment was conducted with monensin in all diets.

We recently reported the time course of the induction of and recovery from classical diet-induced MFD (Rico and Harvatine, 2013). Briefly, MFD was induced to near maximal levels in 9 d by feeding a low-fiber and high-UFA diet (29.5% NDF, 27% starch, and 3.4% C18:2). After correction of both dietary fiber and UFA concentration (36.9% NDF, 18% starch, and 1.6% C18:2), recovery from MFD occurred progressively, as milk fat yield was not different from control on d 11 and was completely recovered by d 19 (Rico and Harvatine, 2013). This correction of both dietary fiber and UFA concentration represents a near best-case scenario for recovery of milk fat, but results in a substantial decrease in diet energy density and risks loss of milk yield.

The objective of the current study was to investigate the effect of dietary NDF and UFA concentration on the time course of recovery of milk fat synthesis and re-establishment of normal rumen FA BH pathways and microbial populations in diets supplemented with monensin. We hypothesized that increasing fermentability (3.4% unit lower NDF) with a similar UFA concentration compared with the control would accelerate recovery of normal rumen BH more than a diet with similar fermentability but higher UFA (2.0% units) compared with the control. This is expected because diet ferment-

ability can alter rumen microbial populations, whereas UFA are the substrates for formation of *trans* FA and also modify rumen microbial populations.

MATERIALS AND METHODS

Experimental Design and Treatments

The experiment was conducted from August to November of 2011. All experimental procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Eight ruminally cannulated and 9 noncannulated multiparous Holstein cows (127 ± 37 d postpartum; mean $\pm$ SD) were randomly assigned to 1 of 6 treatment sequences in a Latin square design (example sequences in Table 1). Cows were housed in a tiestall barn located at the Pennsylvania State University Dairy Production Research and Teaching Center.

Each period was divided into a 10-d induction phase and an 18-d recovery phase (Table 1). During each period, short-term MFD was first induced by feeding a low-fiber, high-UFA diet (induction; 25.9% NDF, 7.1% FA, and 3.3% C18:2; Table 2). The recovery treatments that followed were a diet correcting dietary fiber and UFA (control; 31.8% NDF, 4.2% FA, and 1.65% C18:2); a diet correcting fiber, but not UFA [high oil (**HO**); 31.5% NDF, 6.6% FA, and 2.99% C18:2]; and a diet correcting UFA, but not fiber level [low fiber (**LF**); 28.4% NDF, 4.3% FA, and 1.71% C18:2]. The HO represents higher UFA and LF represents lower fiber relative to the control, respectively. Cows were fed the control diet during a 7-d washout between periods. The combination of the washout and induction provided 17 d between experimental diets and minimized the possibility of carry-over between experimental diets. Monensin (Rumensin 90; Elanco Animal Health, Greenfield, IN) was top-dressed to all cows at a rate of 450 mg/cow per day in 1.0 kg (DM basis) of cookie meal for the duration of the experiment. All cows received bST (Posilac; Elanco Animal Health) every 14 d.

Table 2. Ingredient and chemical composition of experimental diets

Item	Diet ¹			
	Induction	Control	HO	LF
Ingredient, % of DM				
Corn silage ²	36.3	41.1	39.9	35.8
Alfalfa haylage ³	7.7	18.5	19.3	15.6
Canola meal	11.2	11.1	10.5	10.6
Ground corn	18.9	6.6	3.9	15.3
Roasted soybeans	7.5	—	7.8	—
Expeller soybean meal ⁴	0.8	8.8	2.6	6.7
Cottonseed hulls	3.5	3.8	4.8	4.1
Cookie meal	4.6	1.9	1.8	4.3
Grass hay/straw ⁵	3.1	3.1	3.1	2.6
Minerals and vitamins mix ⁶	2.4	3.0	2.8	3.0
Sugar cane molasses	2.1	1.5	1.4	1.4
Soybean oil	1.6	—	1.5	—
Optigen ⁷	0.4	0.5	0.5	0.5
Nutrient, % of DM				
CP	16.3	19.2	19.0	18.0
NDF	25.9	31.8	31.3	28.4
Forage NDF	16.6	22.5	22.4	19.3
ADF	16.3	21.6	21.6	19.0
Starch	31.3	24.0	21.6	29.0
FA	7.1	4.2	6.6	4.3
C18:1n-9	1.53	0.85	1.31	0.92
C18:2n-6	3.30	1.65	2.99	1.71
C18:3n-3	0.42	0.29	0.49	0.26
Mean particle size, ⁹ mm	4.32	3.81	4.19	3.81

¹Induction is a low-fiber and high-unsaturated fatty acid (UFA) diet fed during the induction phase of each period; control is correction of fiber and UFA concentration; high oil (HO) is correction of fiber, but not of UFA concentration; low fiber (LF) is correction of UFA, but not fiber concentration.

²Contained 38.5% DM, 8.1% CP, 31.1% NDF, and 17.3% ADF.

³Contained 46.4% DM, 20.8% CP, 40.9% NDF, and 35.4% ADF.

⁴SoyPLUS (West Central Cooperative, Ralston, IA).

⁵Contained 88.9% DM, 8.6% CP, 68.2% NDF, and 41.3% ADF.

⁶Composition (DM basis): 11% CP; 18% NDF; 5.2% fat; 14.9% Ca; 0.35% P; 4.58% Mg; 0.41% K; 0.31% S; 357 mg/kg of Cu; 1,085 mg/kg of Zn; 181 mg/kg of Fe; 6.67 mg/kg of Se; 262,105 IU/kg of vitamin A (retinyl acetate); 65,421 IU/kg of vitamin D (activated 7-dehydrocholesterol); and 1,970 IU/kg of vitamin E (DL- α tocopheryl acetate).

⁷Optigen is an NPN source (256% CP, DM basis), Alltech Inc. (Lexington, KY).

⁸Analyzed by Cumberland Valley Analytical Services (Maugansville, MD; n = 3 per diet). Fatty acid profile analyzed by GC-flame ionization detection (full dietary FA profile shown in Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2104-8990>).

⁹The particle size distribution was analyzed using the Penn State Particle Separator (n = 2 per diet). Particles remaining (% of total) in the upper sieve, middle sieve, lower sieve, and bottom pan were 3, 27, 47, and 24, respectively, for the induction diet; 3, 35, 41, and 22, respectively, for the control diet; 2, 31, 45, and 21, respectively, for the HO diet; and 3, 28, 45, and 25, respectively, for the LF diet.

Feed Sampling and Analysis

Cows were fed individually once daily (0800 h) at 110% of expected intake and intake was recorded daily. Forage and base diet DM concentration was determined weekly for diet adjustment and DMI determination (72 h in a forced-air oven at 55°C). All individual feed ingredients were sampled weekly, composited by period,

and analyzed as previously described by Rico et al. (2014a).

Milk Sampling and Analysis

Cows were milked twice daily at 0500 and 1700 h, and milk yield was determined by an integrated milk meter (Afimilk; SAE Afikim, Israel). Milk was sampled at both milkings on d 0 (last day of induction phase), 3, 6, 9, 12, 15, and 18 of each recovery phase and analyzed as described by Rico et al. (2014a). Briefly, milk fat was analyzed for fat (filter B) and protein by infrared spectroscopy and FA composition (extracted with hexane:isopropanol, transmethylated in the presence of sodium methoxide, and FAME-quantified by gas chromatography with a capillary column and a flame ionization detector; FA identified are reported in Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-8990>). Under the conditions used, *cis*-9,*trans*-11 CLA was expected to elute with other isomers, including *trans*-7,*cis*-9 CLA and *trans*-8,*cis*-10 CLA; however, *cis*-9,*trans*-11 CLA represents the majority of the peak (Fritsche et al., 1999). The milk FA desaturase indexes were calculated as an estimation of the activity of the stearoyl CoA desaturase enzyme [product/(substrate + product)].

Rumen pH and Microbial Taxa Analysis

Ruminal pH was monitored every 5 min for 24 h on d 0, 3, 6, 9, 12, 15, and 18 of each recovery phase in the 8 ruminally cannulated cows using indwelling ruminal pH probes (Kahne Ltd., Auckland, New Zealand) as described by Ying et al. (2015).

Whole ruminal digesta samples were collected at 1600 h on d 0, 3, 6, 9, 12, 15, and 18 of each recovery period. Samples were stored at -80°C, freeze-dried (Ultra 35-XL; Virtis Co. Inc., Gardiner, NY) and ground in a coffee grinder (model 80335, Hamilton Beach Brands Inc., Glen Allen, VA) before DNA extraction. The DNA was isolated using a commercial kit (QIAamp DNA stool mini kit, Qiagen, Germantown, MD) with modifications similar to Yu and Morrison (2004). Briefly, approximately 220 mg of digesta was homogenized for 5 min with 1.2 mL of lysis buffer and 0.4 g of 0.1-mm sterile zirconia beads using a bench-top vortex equipped with a Mo Bio Vortex-Genie 2 adapter (Mo Bio laboratories, Carlsbad, CA). After bead-beating, samples were incubated twice at 95°C for 15 min with vortexing every 5 min and bead-beating between incubations. The DNA concentration was determined by spectroscopy (NanoDrop ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, DE). The relative abundance of select microbial

taxa was determined using real-time quantitative (q) PCR and 400 nM of previously validated primers and conditions (Supplemental Table S3; <http://dx.doi.org/10.3168/jds.2014-8990>). Reactions were conducted in triplicate (PerfeCTa SYBR Green SuperMix with ROX, Quanta Biosciences, Gaithersburg, MD; Applied Biosystems 7900 HT Fast Real-Time PCR system, Life Technologies, Grand Island, NY). Primer specificity was evaluated by melting curve analysis and efficiency (E) was calculated as $E = 10^{-1/s}$, where s is the slope of standard curve. Abundance of bacteria, protozoa, and fungi are reported as a percent of total bacteria calculated as $E(UB)^{Ct(UB)}/E(TG)^{Ct(TG)}$, where E(UB) is the efficiency of the reaction for the universal bacteria primer set, E(TG) is the efficiency of the reaction for the target gene, and Ct is the average cycle threshold value for each sample. Second, abundance of total fungi, protozoa, and bacteria are reported relative to d 0 of control, when animals were fed a common diet.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS with repeated measures (version 9.3, SAS Institute Inc., Cary, NC) as described by Rico et al. (2014a). Briefly, the model included the random effect of treatment sequence, period, and cow nested in treatment sequence and the fixed effect of treatment, time (day on recovery), the interaction of treatment and time, and the last day of the induction period as a covariate. The preplanned contrasts were control versus HO and control versus LF at each time point. Significance and tendencies were declared at $P < 0.05$ and $P < 0.10$, respectively, for main effects and preplanned contrasts and at $P < 0.10$ and $P < 0.15$, respectively, for interactions.

Second, a random regression analysis was performed on milk fat concentration and yield and concentration of selected milk FA also as described by Rico et al.

(2014a). The model included the random effect of cow and period, and the fixed effect of treatment, linear effect of time, quadratic effect of time, the interaction of treatment and time, and the interaction of treatment and the quadratic effect of time. A reduced model was used which excluded the interaction of treatment by the quadratic effect of time when not significant.

RESULTS

Induction of MFD

Average milk fat concentrations at the start and end of each MFD induction phase averaged 3.10 and 2.26%, respectively, and milk fat yields were 1.30 and 1.01 kg/d (SEM = 0.07 kg/d), respectively (Table 3). This represents a 27 and 22% reduction in milk fat concentration and yield from the start to end of each MFD induction phase. There was no difference in milk fat concentration or yield between treatments at the end of the induction phase of each period or at the end of the washout periods following period 1 and 2 (all $P > 0.2$; data not shown), and milk fat concentration and yield did not differ between the last day of the recovery (d 18 or recovery) and the last day of the washout period (d 0 of induction; Table 3). This indicates little carryover effect entering the induction phase. Chemical composition of all diets closely followed expected values (Table 2) and monensin concentration in the premix topdress was 87% of target value.

DMI and Milk Production and Composition

A treatment by time interaction was observed for DMI ($P = 0.07$; Figure 1a). Dry matter intake sharply decreased in all treatments between d 0 and 1 of the recovery phase and progressively increased thereafter, reaching a plateau on approximately d 7. Dry matter intake was higher in LF than control on 16 of the 18 d

Table 3. Milk yield and composition during the induction phase and end of recovery

Item	Day in induction ¹				Recovery ² d 18	SE	P-value ³
	0	3	7	10			
Milk yield, kg/d							
Milk	41.9 ^{ab}	43.4 ^{ab}	43.0 ^{ab}	44.4 ^a	41.4 ^b	1.52	0.03
Fat	1.30 ^a	1.13 ^{bc}	1.02 ^d	1.01 ^{cd}	1.21 ^{ab}	0.07	<0.001
Milk composition, %							
Fat	3.10 ^a	2.56 ^b	2.32 ^c	2.26 ^c	2.94 ^a	0.14	<0.001

^{a-d}Treatment means with different superscripts differ ($P < 0.05$) by Tukey's test.

¹Milk fat depression was induced by feeding a low-fiber and high-unsaturated fat diet. Samples were taken the day before the diet and after 7 and 10 d.

²Mean of all treatments on the last day of the recovery period.

³Main effect of day.

Figure 1. Effect of primarily correcting dietary fiber or unsaturated fatty acid (UFA) concentration on DMI (A), milk yield (B), and milk fat concentration (C) and yield (D) during recovery from milk fat depression. Treatments were a high-fiber, low-UFA diet (control), a diet correcting UFA, but not fiber level (low fiber; LF), and a diet correcting fiber, but not UFA (high oil; HO). Preplanned contrasts tested the difference between control and LF ($F = P < 0.05$, $F = P < 0.01$, and $f = P < 0.1$) and between control and HO ($O = P < 0.05$, $O = P < 0.01$, and $o = P < 0.1$). Significance of treatment (Trt), time, and the interaction of treatment and time are reported within each figure. The insets in C and D report the slope for the linear (L) and quadratic (Q) coefficients with preplanned contrasts from random regression analysis.

observed and HO was lower than control on d 16. No treatment by time interaction was noted for milk yield, although on d 15 LF was higher than control (4.7 kg/d; $P < 0.01$). An effect of time ($P = 0.04$) and a tendency ($P = 0.09$) for an effect of treatment on milk yield were seen (Figure 1b). Milk yield decreased over time and overall tended to be higher in LF than in control (42.3 vs. 44.2 kg/d).

No treatment by time interactions were observed for milk fat concentration and yield by ANOVA. Milk fat concentration and yield increased progressively in all treatments during the recovery phase (time = $P < 0.001$; Figures 1c and d). Overall, milk fat concentration was lower in LF than in control ($P < 0.05$). Specifically, LF decreased ($P = 0.02$) milk fat concentration compared with control from d 12 to 18 by $9.0 \pm 1.8\%$ (mean $\pm$ SD); however, LF did not affect milk fat yield at any time point. Milk fat concentration and yield were not different between control and HO except on d 15, when HO decreased milk fat yield

by 11% relative to control ($P < 0.03$). By regression analysis, milk fat concentration increased quadratically in all treatments ($P < 0.01$; Supplemental Table S4; <http://dx.doi.org/10.3168/jds.2014-8990>). However, an interaction between treatment and the linear rate of recovery of milk fat concentration was observed (Figure 1), as LF decreased the linear rate of recovery of milk fat concentration compared with control ($P < 0.01$). There were no interactions of treatment and rate or recovery of milk fat yield.

An effect of time on milk protein yield was noted ($P = 0.04$), but no effect of treatment or treatment by time interaction was seen (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2014-8990>). Compared with control, LF increased milk protein yield by $11 \pm 1.5\%$ from d 15 to 18 ($P < 0.05$). However, there was a treatment by time interaction for milk protein concentration ($P < 0.05$; Supplemental Figure S1). Concentration of milk protein progressively decreased from d 3 to 18 in all treatments (time effect: $P < 0.001$); however, from

d 6 to 18 it was $2.9 \pm 0.6\%$ higher in HO than control ($P < 0.05$).

Milk De Novo and Preformed FA

There was a treatment by time interaction for the yield of milk FA less than 16C (de novo synthesized) and 16C FA ($P < 0.001$; Figure 2), as well as for the concentrations of milk de novo, 16C, and greater than 16C (preformed) FA ($P < 0.001$). The concentration of milk de novo FA increased progressively for the control and LF treatments, but was marginally decreased in LF compared with control from d 15 to 18 [$4.3 \pm 0.38\%$ (mean $\pm$ SD); $P < 0.05$; Figure 2a]. Similarly, yield of de novo-synthesized FA progressively increased for both control and LF, but was not different between control and LF at any time point (Figure 2b). In contrast, concentration and yield of de novo-synthesized FA remained relatively constant over time in the HO treatment, and were decreased compared with control from d 3 to 18 (27 ± 1.2 and $34 \pm 3.4\%$ on d 15 and 18, respectively; $P < 0.01$).

Milk 16C FA concentration approached near maximal values by d 3 in both control and LF, but tended to be higher in LF than control on d 3 and 9 ($P = 0.09$). However, milk 16C FA remained constant in HO, and was lower than control from d 3 to 18 ($12 \pm 3.4\%$; $P < 0.001$; Figure 2c). Similarly, yield of 16C FA increased progressively in control and LF, and tended ($P < 0.1$) to be lower in LF than control on d 12; however, it remained relatively constant in HO and was lower than control from d 3 to 18 ($21 \pm 4.5\%$; $P < 0.001$; Figure 2d).

Concentration of milk preformed FA decreased progressively in control and LF and tended to be lower in LF than control on d 6 and 9 ($P < 0.1$; Figure 2e). In contrast, concentration of milk preformed FA slightly increased initially and then remained nearly constant in HO and was on average $21 \pm 5.3\%$ higher than control from d 3 to 18 ($P < 0.01$). Yield of milk preformed FA remained nearly constant in control and LF, but was higher in HO than in control on d 9 and 18 (16 and 12%, respectively; $P < 0.05$; Figure 2f).

Milk Trans FA

There was a treatment by time interaction for the concentration of milk *trans*-10 and *trans*-11 C18:1 and *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA ($P < 0.01$; Figure 3). Concentration of milk *trans*-10 C18:1 and *trans*-10,*cis*-12 CLA decreased progressively in all treatments (Figure 3a and 3c). However, both FA were greatly increased in HO compared with control from d

3 to 18 [*trans*-10 C18:1 increased $140 \pm 50\%$ and *trans*-10,*cis*-12 CLA increased $190 \pm 57\%$ (mean $\pm$ SD)], whereas in the LF treatment both FA were increased compared with control, but near baseline concentration, from d 9 to 18 (*trans*-10 C18:1 increased $67 \pm 25\%$ and *trans*-10,*cis*-12 CLA increased $90 \pm 22\%$).

The concentration of milk *trans*-11 C18:1 decreased modestly over time in control and LF, and was lower in LF relative to control from d 6 to 18 ($30 \pm 2.7\%$; Figure 3b). However, HO increased *trans*-11 C18:1 progressively and was higher than control from d 3 to 18 ($96 \pm 5.5\%$ from d 15 to 18). *Cis*-9,*trans*-11 CLA followed a similar pattern to *trans*-11 C18:1 (Figure 3d). Briefly, *cis*-9,*trans*-11 CLA decreased progressively in both control and LF and did not differ at any time point. In contrast, HO increased progressively and was higher than control from d 3 to 18 ($90 \pm 3.7\%$ from d 15 to 18).

Rate of Recovery of FA

A random regression analysis was used to determine the rate of recovery of selected FA and regression parameters are provided in the supplemental data. Briefly, linear and quadratic interactions with treatment were detected for both the concentration and yield of de novo and 16C FA ($P < 0.01$; Supplemental Table S4; <http://dx.doi.org/10.3168/jds.2014-8990>). Concentration of de novo and 16C FA increased quadratically during recovery in control and LF; however, HO drastically reduced the linear and increased the quadratic rate of recovery compared with control ($P < 0.05$). Linear and quadratic interactions with treatment were also detected for the recovery of preformed FA concentration ($P < 0.001$), as control and LF decreased quadratically and HO increased quadratically. In contrast, only an interaction between treatment and the linear rate of recovery of preformed FA yield was observed ($P = 0.02$). Yield of preformed FA increased linearly in all treatments ($P < 0.001$), but HO increased the rate by approximately 5 fold compared with control ($P = 0.01$).

Interactions between treatment and the linear and quadratic effects of time were observed for the concentrations of milk *trans*-10 C18:1 and *trans*-10,*cis*-12 CLA ($P < 0.001$; Supplemental Table S4; <http://dx.doi.org/10.3168/jds.2014-8990>). For both FA, the linear rate of decrease was reduced by HO and LF compared with control. Additionally, control and LF plateaued near d 12, whereas HO continued to decrease through d 18. Interactions between treatment and the linear and quadratic effects of time were also detected for the concentrations of milk *trans*-11 C18:1 and *cis*-9,*trans*-11 CLA ($P < 0.05$). Both FA increased quadratically in

HO, whereas they decreased quadratically in control and LF. The linear rate of decrease was higher for LF than for control ($P < 0.05$).

A treatment by time interaction was noted for C14 and C16 desaturase indexes (Supplemental Figure S2; <http://dx.doi.org/10.3168/jds.2014-8990>). Both indexes modestly decreased over time, but the C14 index was higher in HO and LF than control from d 6 to 18.

Similarly, the C16 index was higher in HO than control from d 9 to 18, whereas it was higher in LF than control on d 6 and from d 12 to 18 ($P < 0.05$).

Ruminal pH

There was no interaction of treatment and time or overall effect of treatment on rumen average pH and

Figure 2. Effect of primarily correcting dietary fiber or unsaturated fatty acid (UFA) concentration on the concentration and yield of de novo (A and B), 16C (C and D), and preformed (E and F) FA during recovery from milk fat depression. Treatments were a high-fiber, low-UFA diet (control), a diet correcting UFA, but not fiber level (low fiber; LF), and a diet correcting fiber, but not UFA (high oil; HO). Significance of treatment (Trt), time, and the interaction of treatment and time are reported within each figure. Preplanned contrasts tested the difference between control and LF ($F = P < 0.05$, $F = P < 0.01$, and $f = P < 0.1$) and between control and HO ($O = P < 0.05$ and $O = P < 0.01$).

Figure 3. Effect of primarily correcting dietary fiber or unsaturated fatty acid (UFA) concentration on the predominant isomers of the alternate biohydrogenation pathway [*trans*-10 C18:1 (A) and *trans*-10, *cis*-12 CLA (C)] and the predominant isomers of the normal biohydrogenation pathway [*trans*-11 C18:1 (B) and *cis*-9, *trans*-11 CLA (D)] during recovery from milk fat depression. Treatments were a high-fiber, low-UFA diet (control), a diet correcting UFA, but not fiber level (low fiber; LF), and a diet correcting fiber, but not UFA (high oil; HO). Significance of treatment (Trt), time, and the interaction of treatment and time are reported within each figure. Preplanned contrasts tested the difference between control and LF ($F = P < 0.05$, $F = P < 0.01$, and $f = P < 0.1$) and between control and HO ($O = P < 0.05$ and $O = P < 0.01$).

time below pH 5.8 or 5.6. However, an effect of time was observed for all variables. Average rumen pH rapidly increased during the recovery phase in all treatments, peaked on d 3, and modestly decreased thereafter (Figure 4a). The time below pH 5.8 and 5.6 decreased rapidly, reached near a nadir on d 3, and progressively increased from d 3 to 18 in all treatments (Figure 4b and data not shown, respectively).

Ruminal Microbes

The LF diet decreased the relative abundance of *Fibrobacter succinogenes* and tended to decrease *Ruminococcus albus*, whereas HO decreased *Megasphaera elsdenii* (Table 4; Figure 5). A treatment by time interaction was observed for *S. bovis* ($P < 0.05$; Table 4). *Streptococcus bovis* was increased less than 50% on d 6 in LF compared with control and tended to be higher on d 9 and 15. However, *S. bovis* was increased 2 to 5

fold from d 3 to 6 of recovery by HO compared with control.

There was an effect of time on the relative abundance of all microbial groups analyzed during recovery from milk fat depression ($P < 0.05$; Table 4). During recovery *M. elsdenii*, *Selenomonas ruminantium*, and *Prevotella bryantii* were decreased in all treatments and *R. albus*, *F. succinogenes*, *Butyrivibrio/pseudobutyrvibrio*, *Butyrovibrio hungatei*, total fungi, and ciliated protozoa were increased during recovery in all treatments (Figure 5 and 6; Supplemental Figures S3 to S5; <http://dx.doi.org/10.3168/jds.2014-8990>).

DISCUSSION

Alteration of ruminal BH results in classical diet-induced MFD and can be induced by feeding high-concentrate, low-fiber diets (e.g., Peterson et al., 2003); low-fiber, high-oil diets (e.g., Harvatine and Bauman,

2006); or high-fiber, high-oil diets (e.g., AlZahal et al., 2008). In the present study, cows were fed a low-fiber, high-UFA diet for 10 d to induce MFD. Diet UFA were increased using a combination of rapidly available FA from soybean oil and more slowly available FA from roasted soybeans. The predominant FA increased was C18:2, although C18:1 and C18:3 also increased. This short-term treatment reduced milk fat concentration by approximately half of the 50% maximal decrease possible (Bauman and Griinari, 2001), resulted in induction of the fundamental aspects of diet-induced MFD, and provided an experimental model to investigate recovery from the primary causative mechanisms.

In the present study, milk fat concentration before induction and after recovery was lower than typically observed in Holstein cow herds. However, the high-producing cows used in the experiment were expected to be lower in milk fat concentration (Rico et al., 2014c), and *trans* isomer concentration indicated a modest presence

of alternate BH intermediates by the end of the recovery phase, suggesting some degree of MFD. Importantly, the objective of the current experiment was to investigate the role of individual diet components on the rate of recovery, which does not require maximal induction nor complete recovery of diet-induced MFD. We have previously observed similar timing of recovery between experiments with varying levels of milk fat (Rico and Harvatine, 2013; Rico et al., 2014a,b).

The induction diet is representative of a high-fermentability, high-UFA diet sometimes unknowingly fed on farms due to changes in forage DM, nutrient composition, and mixing errors, which lead to diet-induced MFD. Additionally, it is also representative of smaller decreases in milk fat observed when diets are marginally high in UFA or fermentability. A short-term induction period was selected because it represents the causative factors of diet-induced MFD and low milk fat is normally rapidly recognized on farms. Longer induction periods may actually result in secondary adaptations and cloud the investigation of the primary mechanisms.

The recovery diets in the current experiment were designed relative to each other and not the MFD induction diet, which was simply used to achieve short-term diet-induced MFD. The control diet corrected both fermentability and UFA level, the HO diet predominantly corrected fermentability while maintaining high UFA, whereas the LF diet predominantly corrected UFA level while maintaining high fermentability. The recovery diets were selected to represent applicable diets that may be fed and that are expected to provide at least partial recovery from MFD. Diets balanced relative to the induction diet may have failed to provide recovery and may not have represented applicable diets. Importantly, a key aspect of the experiment focuses on the time course of recovery with diets differing in fermentability and UFA. Correction of UFA was achieved in the control diet by the removal of soybean oil and substitution of expeller soybean meal for whole roasted soybeans; diet fermentability was corrected by increasing dietary NDF 5.9 percentage units, which reduced starch by 7 percentage units (corn grain substituted by forages). Compared with the control, the effect of dietary fat was tested by increasing total FA and C18:2 by 2.4 and 1.34 percentage units (DM basis), respectively, in the HO diet. The effect of fermentability was tested by a forage-to-grain substitution that decreased dietary NDF 3.4% units (DM basis) in LF relative to control.

The treatments were selected to represent substantially different, but real-world applicable diets. Altered ruminal biohydrogenation is a result of the rumen environment, carbohydrate and FA profile and availability, and many complex interactions (Bauman and Griinari,

Figure 4. Effect of primarily correcting dietary fiber or unsaturated fatty acid (UFA) concentration on average pH (A) and time below pH 5.8 (B). Treatments were a high-fiber, low-UFA diet (control), a diet correcting UFA, but not fiber level (low fiber; LF), and a diet correcting fiber, but not UFA (high oil; HO). Significance of treatment (Trt), time, and the interaction of treatment and time are reported within each figure.

Table 4. Effect of primarily correcting dietary fiber or unsaturated fatty acid (UFA) concentration on the abundance of select ruminal microbes during recovery from diet induced milk fat depression in monensin supplemented diets

Item, ¹ % of total bacteria	Treatment ^{2,3}			SEM	P-value ⁴		
	Control	HO	LF		Trt	Time	Trt × time
Lactate utilizing							
<i>Megasphaera elsdenii</i>	0.020	0.009**	0.013	0.009	0.12	<0.01	0.15
<i>Selenomonas ruminantium</i>	0.530	0.463	0.573	0.054	0.07	<0.01	0.76
Fibrolytic							
<i>Ruminococcus albus</i>	0.025	0.028	0.019†	0.037	0.06	<0.01	0.61
<i>Fibrobacter succinogenes</i>	0.466	0.495	0.385*	0.103	<0.01	<0.01	0.16
<i>Prevotella ruminicola</i>	0.756	0.831	0.740	0.085	0.49	<0.01	0.99
Amylolytic							
<i>Streptococcus bovis</i>	0.0005	0.0024**	0.0008*	0.0006	<0.01	<0.01	<0.01
<i>Prevotella bryantii</i>	0.247	0.234	0.271	0.355	0.51	<0.01	0.19
Trans-11 C18:1-producing							
<i>Butyrivibrio/Pseudobutyvibrio</i>	0.314	0.341	0.306	0.398	0.74	<0.01	0.69
<i>Butyrivibrio hungatei</i>	0.0216	0.0220	0.0193	0.0259	0.76	0.02	0.60
Total fungi	0.237	0.299	0.214	0.057	0.21	<0.01	0.53
Ciliate protozoa	2.68	2.83	3.09	0.83	0.56	0.02	0.56
Relative to d 0 ⁵							
Bacteria	2.16	2.29	2.20	0.14	0.61	<0.01	0.91

¹Relative abundance (% of target gene relative to total bacterial 16S rDNA).

²Treatments during recovery from diet induced milk fat depression: control = high-fiber, low-oil diet; HO = high-fiber, high-oil diet; LF = low-fiber, low-oil diet.

³Preplanned contrasts tested the difference between control and LF (* $P < 0.05$, † $P < 0.1$), and between control and HO (** $P < 0.01$).

⁴Trt = treatment effect; Time = effect of time; and Trt × time = treatment by time interaction.

⁵Data standardized to average of all treatments on d 0 of recovery.

2001; Lourenço et al., 2010). It is important to recognize that treatments are expected to have an effect on the rumen environment, microbial populations, and amount of substrate available for biohydrogenation.

All recovery diets contained higher NDF than the induction diet, which may explain the initial reduction in intake at the start of the recovery phase. The LF diet was lower in NDF and forage NDF than the control diet, and a change in DMI is expected depending on ruminal fill and energy balance (Allen, 2000). Rumen pH did not differ between treatments. However, abundance of *R. albus* and *F. succinogenes* was moderately lower in LF, reflecting the negative effect of the more fermentable diet on fibrolytic bacteria. Similarly, the lower fermentability in the recovery diets relative to the induction phase was reflected by the reductions in lactate using bacteria *Megasphaera elsdenii* and *S. ruminantium* (Counotte et al., 1981), and in amylolytic bacteria such as *P. bryantii*. Dry matter intake was unaffected by HO compared with control, despite the higher dietary oil concentration and energy density in the HO diet. This difference may have been too small to observe a change in intake, and although high UFA concentrations in the diet are commonly expected to impair fiber digestibility through negative effects on fibrolytic bacteria (Jenkins, 1993; Maia et al., 2007), effects were not observed on *R. albus*, *F. succinogenes*, or *Prevotella ruminicola* in the current experiment. Ru-

men pH recovered rapidly in all treatments, but milk *trans* isomer profiles clearly indicated altered fermentation with HO, which could be the result of microbial populations shifts in the rumen. Unsaturated FA are known to have direct toxic effects on ruminal bacteria (Maia et al., 2010, 2007), resulting in a shift in the ruminal microbial population (Lourenço et al., 2010). Interestingly, *S. bovis* abundance was progressively increased by HO and was increased to a lesser extent by LF relative to control, suggesting a positive effect of UFA on this species, perhaps related to its moderately higher tolerance to UFA compared with other amylolytic bacteria such as *P. bryantii* (Maia et al., 2007).

Previously, we conducted a high-frequency time course and reported that correction of both dietary fiber and UFA concentration recovered milk fat concentration and yield within 19 d (Rico and Harvatine, 2013). In the current study, a similar progressive increase in milk fat yield was observed over 18 d by minimizing UFA or increasing fiber and minimizing UFA. Milk fat concentration was lower in the LF diet than control from d 12 to 18; however, milk yield was numerically increased during the same period, resulting in no change in milk fat yield. Differences in the concentration and yield of de novo-synthesized FA was minimal between control and LF, and both followed a similar trend, increasing progressively during recovery from diet-induced MFD and plateauing around d 15. Interestingly, *trans*-10 18:1

and *trans*-10,*cis*-12 CLA decreased rapidly in LF during the first 6 d of recovery, but were higher than control for the remainder of the recovery phase, coinciding with the same period of reduced milk fat concentration suggesting some maintenance of the alternate BH pathways and inability to biohydrogenate the high level of UFA in the diet.

Although increasing fiber, but not correcting UFA concentration, resulted in lower milk fat yield on d 15, it was not different from control during the rest of the recovery phase. However, de novo FA concentration and yield did not recover in the HO treatment, which is likely a combined effect of both higher milk *trans*-10 18:1 and *trans*-10,*cis*-12 CLA concentrations in the HO treatment and higher availability of preformed FA, both of which can reduce de novo FA in milk fat (Rico et

al., 2014c). Although *trans*-10 18:1 and *trans*-10,*cis*-12 CLA decreased progressively during recovery, they were considerably higher in HO than control. The progressive increase in milk *trans*-11 C18:1 and *cis*-9,*trans*-11 CLA in HO indicated a gradual return to normal ruminal BH. Long-chain FA are known to decrease de novo-synthesized FA in milk (Drackley et al., 1992). In the current experiment, the concentration of FA in the HO diet was more than 2.1 percentage units higher than control and LF diets, but was 0.5 percentage units lower than the MFD-induction diet and the reduction in intake further decreased absorbed FA. Interestingly, the yield of de novo FA was initially numerically decreased during recovery indicating little effect of the slight reduction in absorbed FA. The observed increase in milk preformed FA yield in the HO group was likely

Figure 5. Effect of primarily correcting dietary fiber or unsaturated fatty acid (UFA) concentration on the abundance of *Megasphaera elsdenii* (A), *Ruminococcus albus* (B), *Streptococcus bovis* (C), and *Fibrobacter succinogenes* (D) expressed as a percent of total bacteria. Treatments were a high-fiber, low-UFA diet (control), a diet correcting UFA, but not fiber level (low fiber; LF), and a diet correcting fiber, but not UFA (high oil; HO). Significance of treatment (Trt), time, and the interaction of treatment and time are reported within each figure. Preplanned contrasts tested the difference between control and LF ($F = P < 0.05$, $F = P < 0.01$, and $f = P < 0.1$) and between control and HO ($O = P < 0.05$, $O = P < 0.01$, and $o = P < 0.1$).

Figure 6. Change in ruminal abundance (% of total bacteria) of *Selenomonas ruminantium* and *Prevotella ruminicola* (A), *Butyrivibrio*/*Pseudobutyrvibrio*, total fungi, and *Prevotella bryantii* (B), and ciliate protozoa and *Butyrivibrio hungatei* (C) during recovery from milk fat depression. The effect of time was significant for all species, but no treatment by time interactions (*P*-values and SEM in Table 4) were noted. The least squares means of time are shown.

the result of increased absorbed FA and indicated little inhibition of the uptake of preformed FA in the mammary gland.

Megasphaera elsdenii (YJ4) was reported to be a *trans*-10,*cis*-12 CLA producer (Kim et al., 2002); however, in the present experiment, no apparent relationship was noted between *trans*-10,*cis*-12 CLA and *M. elsdenii*, similar to other reports (Wallace et al., 2006). In contrast, *S. bovis* was increased by HO, but it is traditionally known as an amylolytic bacteria that produces lactic acid (Russell and Hino, 1985; Cotta, 1988). It is not known to be involved in the formation of *trans*-10,*cis*-12 CLA, but is associated with the formation of hydroxy-C18 FA (Maia et al., 2007). The *Butyrivibrio*/*Pseudobutyrvibrio* and *B. hungatei* groups are known for their role in the formation of *cis*-9,*trans*-11 CLA and *trans*-11 C18:1 from LA (Lourenço et al., 2010), and both reached peak concentrations by d 6 of recovery regardless of treatment. Similar recovery trends were observed for fungi and protozoa. Ciliate protozoa are not known to have important roles on ruminal metabolism of LA, but contribute to the ruminal outflow of both *cis*-9,*trans*-11 CLA and *trans*-11 C18:1 (Devillard et al., 2006; Yáñez-Ruiz et al., 2006). Conversely, some species of anaerobic fungi, such as *Neocallimastix frontalis*, produce *cis*-9,*trans*-11 CLA. The major changes in total fungi during recovery highlights the potential for other roles in rumen biohydrogenation.

A 2-phase response previously reported on BH intermediates during recovery from diet-induced MFD by correction of dietary fiber and UFA (Rico and Harvatine, 2013; Rico et al., 2014b). Briefly, milk concentrations of intermediates in the altered BH pathway (*trans*-10 C18:1 and *trans*-10,*cis*-12 CLA) decreased during the first phase and presumably represent re-establishment of normal microbial populations. The second phase was characterized by a slight elevation of *trans* FA in the normal pathway (*trans*-11 C18:1 and *cis*-9,*trans*-11 CLA) suggesting a secondary phase of adaptation in ruminal microbial populations and BH capacity. In the current experiment, FA in the altered pathway decreased rapidly during the first phase of recovery for control and LF treatments, but the isomers in the normal pathway were not elevated above baseline in a secondary phase. It is possible that monensin supplementation in the current experiment prevented or delayed the second adaptation in BH intermediates observed previously previous experiments (Rico and Harvatine, 2013; Rico et al., 2014b). Indeed, the major adaptations of ruminal microbes observed in the present experiment occurred during the initial phase of recovery, and no major changes were observed for most taxa after d 6 of recovery.

Diet-induced MFD is correlated with changes in the rumen environment, microbial populations, and amount and type of FA and carbohydrate substrate available. Low rumen pH has been classically proposed as a driving factor in the change in the microbial population and rates and pathway of BH, and has been clearly demonstrated in vitro (Choi et al., 2005; Fuentes et al., 2009). Additionally, rumen pH is correlated with milk fat concentration in vivo (Allen, 1997). However, in the current experiment, rumen pH was corrected rapidly when fed the recovery diets, but the change in absorbed BH intermediates occurred progressively in all treatments. Additionally, rumen pH was corrected in the HO treatment, but recovery of normal BH intermediates in milk was inhibited, which could reflect the higher amount of substrate in addition to difference in passage rate and BH capacity. The lag between improvement in rumen pH and recovery of normal BH intermediates may demonstrate the lag required for re-establishment of the microbial populations, or may represent a smaller role of pH in diet-induced MFD than ruminal UFA load.

CONCLUSIONS

Decreasing alternate BH intermediates in milk fat following classical diet-induced MFD can most effectively be achieved through correction of dietary UFA concentration. Although recovery of milk fat yield was not different among treatments, increasing dietary fiber while maintaining a high UFA concentration (4.8%) resulted in increased *trans*-10 C18:1 and *trans*-10,*cis*-12 CLA, which is associated with classical diet-induced MFD. The high-fermentability diet (3.4% lower in NDF) also slightly increased these BH intermediates above control. Overall, milk fat synthesis was rapidly increased by decreasing diet fermentability and UFA concentration, which provides support for focusing on these risk factors when designing strategies to recover from diet-induced MFD.

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