Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) on risk of biohydrogenation-induced milk fat depression

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ABSTRACT

Diet-induced milk fat depression (MFD) is a multifactorial condition resulting from the interaction of numerous risk factors, including diet fermentability and unsaturated fatty acids concentration, feed additives, and individual cow effects. 2-Hydroxy-4-(methylthio)butanoate (HMTBa) is a methionine analog that has been observed to increase milk fat in some cases, and interactions with MFD risk factors may exist. The objective was to evaluate the effect of HMTBa supplementation on milk fat synthesis in cows with different levels of milk production and fed diets with increasing risk of biohydrogenation-induced MFD. Sixteen high-producing cows (44.1 ± 4.5 kg of milk/d; mean $\pm$ SD) and 14 low-producing (31.4 ± 4.3 kg of milk/d) were used in a randomized block design. Treatments were unsupplemented control and HMTBa fed at 0.1% of diet dry matter (25 g/d at 25 kg of dry matter intake). The experiment was 70 d and included a 14-d covariate period followed by 3 phases whereby diets were fed with increasing risk of MFD to determine the interaction of treatment and diet-induced MFD. During the low-risk phase, the base diet was balanced to 33.5% neutral detergent fiber (NDF) and had no exogenous oil (28 d); during the moderate-risk phase, the diet was balanced to 31% NDF and contained 0.75% soybean oil (14 d); and, during the high-risk phase, the diet was balanced to 28.5% NDF and contained 1.5% soybean oil (14 d). An interaction of treatment, production-level, and dietary phase was observed. Low producing cows neither experienced substantial biohydrogenation-induced MFD nor a response in milk fat to HMTBa supplementation. In high-producing cows, HMTBa maintained higher milk fat concentration during the moderate- (2.94 vs. 3.49%) and high-risk (2.38 vs. 3.11%) phases. High-producing

cows receiving HMTBa also had greater milk fat yield (0.94 vs. 1.16 kg/d) and lower *trans*-10 C18:1 (6.11 vs. 1.50) during the high-risk phase. In conclusion, HMTBa increased milk fat in situations with a high risk of biohydrogenation-induced MFD by decreasing absorption of alternate biohydrogenation intermediates.

Key words: 2-hydroxy-4-(methylthio)butanoate, biohydrogenation, milk fat, milk fat depression

INTRODUCTION

Fat is one of the most important components of milk, as it significantly affects the value of milk, the yield and quality of dairy products, and because of the recent associations between consumption of milk bioactive fatty acids (FA) and human health (Bainbridge et al., 2016). Consequently, the mechanisms regulating milk fat synthesis in the mammary gland, as well as nutritional and management strategies that improve milk fat synthesis, have been studied extensively. Low-milk fat syndrome, contemporarily called diet-induced milk fat depression (MFD), is a condition in which milk fat yield decreases up to 50% with generally no change in yields of milk and other milk components (Harvatine et al., 2009b). Milk fat synthesis is affected by several factors (e.g., genetics, physiological state, and environment), but is especially responsive to nutrition. The biohydrogenation (BH) theory (Bauman and Grinari, 2003) of MFD is a unifying concept that mechanistically explains the inhibition of milk fat synthesis when feeding highly fermentable and high-UFA diets. This model attributes the causal mechanism to changes in ruminal BH pathways, leading to increased formation and rumen outflow of specific bioactive FA that inhibit mammary lipid synthesis.

The understanding of dietary regulation of milk fat synthesis has improved, yet varying levels of MFD still commonly occur on dairy farms. This is primarily because MFD is a multifactorial condition resulting from the interaction of several factors, such as dietary FA level, FA profile and availability, diet fermentability, feeding strategies, rumen modifiers, and individual cow effects (Harvatine, 2017). Diet UFA level and

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carbohydrate fermentability have been characterized as central risk factors for BH-induced MFD (Rico and Harvatine, 2013; Rico et al., 2014a). Additionally, an association between level of milk production and risk of BH-induced MFD has been proposed. For instance, supplementation with calcium salts of UFA decreased milk fat in high-producing cows, whereas no MFD was observed in the low-producing cows (Harvatine and Allen, 2006; Rico et al., 2014b).

Varying levels of evidence support the effectiveness of some rumen modifiers in attenuating BH-induced MFD. Early work observed that the methionine analog, 2-hydroxy-4-(methylthio)butanoate (**HMTBa**), increased milk fat when feeding highly fermentable diets (Rosser et al., 1971; Huber et al., 1984). A recent meta-analysis observed increased milk fat yield with HMTBa supplementation (Zanton et al., 2014), but it was inconclusive on the role of HMTBa in circumstances of MFD. As BH-induced MFD is the result of the interaction of numerous factors, studying one single factor at a time will be less informative and more inconsistent than studying multiple factors simultaneously. The current study was conducted to evaluate the interaction between dietary risk factors (UFA and carbohydrate fermentability), cow milk production level, and supplementation with a rumen modifier (HMTBa). The objective was to evaluate the effect of HMTBa supplementation on milk fat synthesis in 2 groups of cows with different levels of milk production and fed diets with increasing risk of BH-induced MFD. Our hypothesis was that high-producing cows are at higher risk of BH-induced MFD and that HMTBa would reduce the extent of BH-induced MFD.

MATERIALS AND METHODS

Experimental Design and Treatments

All experimental procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. The experiment was conducted from October to December 2013 at the Pennsylvania State University Dairy Production Research and Teaching Center (University Park). Thirty multiparous Holstein cows were used in a randomized block design that tested the effect of treatment during 3 dietary phases that progressively increased risk for diet-induced MFD. Animals were housed individually in tiestalls with rubber mattresses and sawdust bedding and had continuous access to water. Cows were blocked by milk production (high or low) at the end of a 14-d pretrial period (Table 1). The high-producing group ($n = 16$; 166 ± 69 d postpartum; mean $\pm$ SD) averaged 44.1 ± 4.5 kg/d of milk and the low-producing group

($n = 14$; 267 ± 82 d postpartum) averaged 31.4 ± 4.3 kg/d of milk. Cows were paired within block (high and low) and randomly assigned to 1 of the 2 treatments: control (**CON**) or HMTBa (0.1% of diet DM, targeting 25 g of HMTBa per cow/d at 25 kg of DMI; Table 2). The HMTBa (Alimet, Novus International Inc., St. Charles, MO) was provided in a corn carrier and mixed in the TMR. An equivalent amount of the same ground corn carrier was added to the control treatment. The experiment was split into 3 phases that fed diets formulated to have low, moderate, and high risk of BH-induced MFD, respectively (Table 3). Risk for altered BH was increased by reducing diet NDF and increasing UFA and starch content (Table 3). Diet UFA were increased using a combination of rapidly available FA from soybean oil and more slowly available FA from roasted soybeans. The low-risk phase was 28 d to allow complete adaptation of the rumen to HMTBa, and the moderate- and high-risk phases were 14 d, as previous time course work has demonstrated that changes in BH occur within 10 to 14 d (Rico and Harvatine, 2013). Importantly, phase is the repeated variable and the effect of treatment is only compared within phase, as phase and treatment are confounded. Diets were fed as a TMR once daily at 0700 h at 110% of expected daily intake. Cows were treated with rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 14 d.

Sampling and Measurements

Feed intake was measured daily. Cows were milked twice daily at 0500 and 1700 h and milk yield determined by an integrated milk meter (AfiMilk, SAE Afikim, Afikim, Israel). The parlor was calibrated using a stall deviation calculated using data from the entire herd (>200 cows) over 7 d. Stall deviations were determined by modeling the effect of day, milking (a.m./p.m.), cow, and stall, excluding observations of experimental cows during treatment periods. Milk was sampled at both milkings once per week and was composited based on yield at each milking. Body weight was recorded as cows exited the parlor throughout the trial (AfiFarm 3.04E scale system, SAE Afikim). Feed ingredients were sampled once per week and stored at -20°C , thawed at room temperature, dried at 55°C in a forced-air oven for 72 h, and ground in a Wiley mill through a 1-mm screen (A. H. Thomas, Philadelphia, PA). Feed samples were composited within dietary phase (equal dry weight basis). Blood samples were collected from the tail vein using potassium EDTA vacuum tubes (Greiner Bio-One North America Inc., Monroe, NC) at 0600, 1400, and 2000 h. Blood was immediately placed on ice, centrifuged within 30 min at $1,300 \times g$ for 15 min at 4°C , and plasma was harvested

Table 1. Characteristics of cows in the low- and high-production block at the end of the pretrial period

Item	Block ¹		SEM	P-value
	Low	High		
DIM	267	166	19.4	<0.01
DMI (kg/d)	24.7	26.8	0.62	0.02
Milk yield (kg/d)	31.4	44.1	1.30	<0.001
Milk fat (%)	3.94	3.20	0.16	<0.01
Milk fat (kg/d)	1.12	1.37	0.06	0.01
Milk protein (%)	3.40	3.26	0.08	0.22
Milk protein (kg/d)	0.97	1.40	0.04	<0.001
Milk FA, g/100 g of total FA ²				
<i>trans</i> -10 C18:1	0.48	0.77	0.12	0.11
Σ <16 C	25.4	25.8	0.62	0.57
Σ 16 C	25.9	26.9	0.52	0.20
Σ >16 C	41.1	39.2	0.83	0.13

¹Milk production level at the end of the pretrial period (low = 14 cows, high = 16 cows).

²Fatty acids (FA) <16 C originate from de novo synthesis in the mammary gland; FA >16 C originate from extraction from plasma, and 16 C FA originate from both sources.

and stored at -20°C until laboratory analysis. Prior to analysis, plasma samples from 1400 and 2000 h were composited by cow to represent a postabsorptive state.

Sample Analysis

Feed samples were analyzed for DM, CP, and starch by wet chemistry procedures according to AOAC International (2000) and for NDF and ADF according to Van Soest et al. (1991). Total FA concentration and FA profile of feed samples was determined by GC after direct methylation (Sukhija and Palmquist, 1988), as described by Rico and Harvatine (2013). Plasma samples were analyzed for insulin (Coat-a-count insulin kit, Siemens Healthcare Diagnostics, Los Angeles, CA), glucose (PGO Enzyme procedure no. P 7119, Sigma-Aldrich, St. Louis, MO), BUN [Modified Enzymatic Urea Nitrogen (Procedure No. 2050); Stanbio Laboratory, Boerne, TX], and FA (Wako HR Series NEFA-HR kit, Wako Chemicals USA Inc., Richmond, VA), as modified by Ballou et al. (2009).

One milk sample was stored at 4°C with preservative (Bronolab-WII, Advanced Instruments Inc.,

Norwood, MA) until analyzed for fat and protein by Fourier transform infrared spectroscopy (Fossomatic 4000 Milko-Scan and 400 Fossomatic, Foss Electric, Hillerød, Denmark; at Dairy One Laboratory, Ithaca, NY). A second milk sample was immediately centrifuged at $3,000 \times g$ for 15 min at 4°C and fat cake was stored at -20°C before analysis of FA composition, as described by Rico and Harvatine (2013). In short, lipid extraction was performed according to Hara and Radin (1978) using hexane:isopropanol. Fatty acid methyl esters were prepared by base-catalyzed transmethylation according to Chouinard et al. (1999). Fatty acid methyl esters were quantified by GC using an Agilent 6890A gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a fused-silica capillary column (SP-2560, $100 \text{ m} \times 0.25 \text{ mm i.d.}$ with $0.2\text{-}\mu\text{m}$ film thickness; Supelco Inc., Bellefonte, PA) and a flame ionization detector with hydrogen as the carrier gas. Initial oven temperature was 80°C , which was increased by $2^{\circ}\text{C}/\text{min}$ to 190°C and held for 15 min. Inlet and detector temperatures were 250°C with a 100:1 split ratio. Constant gas flows were 1 mL/min for hydrogen carrier, 25 mL/min for detector hydrogen, 400 mL/min for detector

Table 2. Overview of experimental design

Item		BH-induced MFD risk ¹		
		Low, d 0 to 28	Moderate, d 29 to 42	High, d 43 to 56
Block ²	Low	Control HMTBa ³	Control HMTBa	Control HMTBa
	High	Control HMTBa	Control HMTBa	Control HMTBa

¹Risk of biohydrogenation (BH)-induced milk fat depression (MFD).

²Milk production level: low-producing cows (low) averaged $31.4 \pm 4.3 \text{ kg/d}$ and high-producing cows (high) averaged $44.1 \pm 4.5 \text{ kg/d}$ at the end of the pretrial period.

³HMTBa = 2-hydroxy-4-(methylthio)butanoate.

airflow, and 40 mL/min for detector nitrogen plus carrier. Fatty acid peaks were identified using FAME standards (GLC 461, GLC 780, and pure *trans*-10,*cis*-12 CLA and *cis*-9,*trans*-11 CLA, NuChek Prep Inc., Elysian, MN; Bacterial Acid Methyl Ester Mix, 47080-U, Sigma-Aldrich; and GLC 110 mixture, Matreya LLC., State College, PA). Recovery of individual FA were determined using an equal weight reference standard (GLC 461; NuChek Prep Inc.). Correction factors for individual FA and calculation of milk FA yield were carried out as described by Rico and Harvatine (2013).

Statistical Analysis

Data were analyzed as a randomized block design using PROC MIXED of SAS with repeated measures (version 9.3; SAS Institute Inc., Cary, NC). An interaction of treatment, production level (block), and experimental day (dietary phase) was observed; thus, data were analyzed within block (high or low) with the following reduced model

$$Y_{ijkl} = \mu + T_i + C_j(T_i) + D_k + T_i D_k + \beta_l + \varepsilon_{ijkl},$$

where Y_{ijkl} is the variable of interest, μ is the overall mean, T_i is the fixed effect of treatment (control or HMTBa), $C_j(T_i)$ is the random effect of cow nested within treatment i , D_k is the fixed effect of experimental day ($k = 1$ to 8), $T_i D_k$ is the interaction of treatment and experimental day, β_l is the fixed effects of pretrial measurements (covariate), and ε_{ijkl} is the residual error. The ARH(1) or AR(1) covariance structures were used depending on model fit and the Kenward-Roger denominator degrees of freedom adjustment was employed. The preplanned contrast tested the effect of HMTBa at each day. In the time-course data, the treatment effect was similar within dietary phase (low, moderate, and high risk of MFD); therefore, for simplicity, effect of treatment was tested by 2-wk intervals that matched dietary phases. Data points with Studentized residuals outside of ± 3.0 were considered outliers and excluded from analysis, which typically included less than 4 data points per variable. Significant differences were

Table 3. Ingredient and chemical composition of basal diets with increasing risk of biohydrogenation (BH)-induced milk fat depression (MFD)

Item	Risk of BH-induced MFD		
	Low	Moderate	High
Ingredient (% of DM)			
Corn silage	40.5	38.1	35.0
Alfalfa haylage	14.7	13.6	12.5
Processed grass hay	2.9	2.7	2.5
Ground corn	7.7	12.3	17.2
Canola meal	11.6	11.1	10.9
Cottonseed hulls	4.1	3.5	2.8
Roasted soybeans ¹	3.5	5.6	6.6
Minerals and vitamins mix ²	3.3	3.3	3.3
Soybean oil	0.0	0.7	1.5
Corn gluten meal	4.3	4.2	4.0
Soybean hulls	5.0	2.5	1.4
Molasses	2.5	2.4	2.3
Composition (% of DM, unless noted)			
CP	17.1	17.3	17.3
RDP (% of CP)	62.0	61.6	61.1
ADF	23.5	21.2	19.3
NDF	33.9	30.9	28.5
Forage NDF	20.2	18.3	17.5
Starch	24.4	26.8	29.2
Fatty acids	4.1	4.9	5.6
PUFA	2.2	2.7	3.1
MUFA	0.9	1.1	1.2
peNDF ³	19.0	17.3	15.1

¹Whole soybeans were roasted in a mobile roaster and were approximately 158°C when removed from the roaster. Beans were rolled into approximately quarters before feeding.

²Composition (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; 65,421 IU/kg of vitamin D; and 1,970 IU/kg of vitamin E (Cargill Animal Nutrition, Roaring Spring, PA).

³peNDF = physically effective NDF. Analyzed using the Penn State Particle Separator (Nasco, Fort Atkinson, WI). Particles remaining (% of total) in the upper sieve, middle sieve, lower sieve, and bottom pan were 3, 37, 16, and 44, respectively, for the low-risk diet; 3, 35, 18, and 44, respectively, for the moderate-risk diet; 3, 35, 15, and 47, respectively, for the high-risk diet.

Table 4. Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) on performance and milk composition in high- and low-producing cows

Item (kg/d, unless noted)	Block ¹	Treatment (Trt) ²			<i>P</i> -value ³		
		Control	HMTBa	SEM	Trt	Day	Trt × day
DMI	H	27.3	25.6	0.74	0.10	<0.01	0.53
	L	24.5	24.1	0.86	0.70	<0.01	0.60
Milk yield	H	40.8	39.5	3.85	0.74	0.01	0.47
	L	21.5	23.8	1.34	0.24	<0.01	0.81
Milk fat (%)	H	2.98	3.42	0.10	<0.01	<0.01	<0.05
	L	3.94	3.96	0.09	0.92	0.02	0.46
Milk fat	H	1.26	1.27	0.05	0.87	<0.01	0.02
	L	0.80	0.98	0.07	0.10	<0.01	0.96
Milk protein (%)	H	3.33	3.36	0.08	0.46	<0.01	0.64
	L	3.63	3.52	0.07	0.05	<0.01	0.58
Milk protein	H	1.40	1.28	0.06	0.17	0.54	0.38
	L	0.73	0.85	0.04	0.06	<0.01	0.16

¹Milk production level: high-producing cows (H) averaged 44.1 ± 4.5 kg/d and low-producing cows (L) averaged 31.4 ± 4.3 kg/d at the end of the pretrial period.

²Treatment was control (ground corn) or HMTBa (0.1% of diet DM provided in a ground corn carrier).

³Effect of treatment (Trt), experimental day, and their interaction.

declared at $P < 0.05$ for main effects and $P < 0.10$ for interactions and tendencies were declared at $P < 0.10$ for main effects and $P < 0.15$ for interactions.

RESULTS

Effect of Production Level

Milk yield and composition differed between the low- and high-producing blocks at the end of the pretrial period (Table 1). Low-producing cows had 95 d greater DIM, 2.1 kg/d lower DMI, and 12.7 kg/d lower milk yield compared with high-producing cows (Table 1). Additionally, low-producing cows had 0.74 percentage points higher milk fat concentration, but had lower yield of milk fat (248 g/d; $P = 0.01$) and milk protein (427 g/d; $P < 0.001$). No difference between the 2 blocks was observed for FA by source (de novo, mixed source, and preformed), but *trans*-10 C18:1 was numerically higher in high-producing cows (0.77 vs. 0.48; $P = 0.11$; Table 1).

Performance Responses

Body weight was not affected by treatment and averaged 740 ± 30 kg in the high-producing group and 703 ± 19 kg in the low-producing group ($P = 0.90$ and $P = 0.70$, respectively). Dry matter intake, milk yield, and milk protein concentration and yield were not affected by treatment in either block (Table 4). Moreover, no treatment by day interaction was observed for any of these variables (Table 4). A tendency for higher milk protein concentration (3.63 vs. 3.52%, $P = 0.05$) in low-producing cows in the control treatment was observed.

We found no effect of treatment or treatment by day interaction for milk fat concentration and yield in the low-producing group (Table 4). Milk fat concentration remained relatively constant and milk fat yield decreased slightly over the 3 dietary phases in low-producing cows on both treatments (Figure 1A and B). On the other hand, a treatment by day interaction was observed for milk fat concentration and yield in the high-producing group (Table 4). In the high-producing cows, milk fat concentration did not differ between treatments during the low-risk phase; but, during the moderate- and high-risk phases, control cows progressively decreased milk fat content, whereas HMTBa cows maintained higher milk fat concentration (0.55 and 0.73 percentage points, respectively; Figure 1A and Supplemental Table S1, <https://doi.org/10.3168/jds.2017-13446>). Milk fat yield did not differ between treatments during low and moderate risk phases, but was higher by 220 g/d in HMTBa during the high-risk phase (Supplemental Table S1 and Figure 1B).

No overall effect of treatment was observed on the concentration of BUN, nonesterified FA, glucose, and insulin in plasma of high- or low-producing cows in either fasting or postabsorptive state ($P > 0.12$ for all; Supplemental Table S2, <https://doi.org/10.3168/jds.2017-13446>). The treatment by time by production level interaction was not significant for the plasma variables ($P > 0.50$ for all).

Milk FA

We observed no overall effect of treatment or treatment by day interactions for *trans*-11 C18:1, *trans*-10 C18:1, FA by source (de novo, mixed, and preformed),

and total odd- and branched-chain FA (OBCFA) in the low-producing block (Table 5). In the high-producing cows we found a treatment by day interactions for *trans*-11 C18:1, *trans*-10 C18:1, and sum of de novo synthesized FA (<16 C; Table 5). During the high-risk phase, high-producing cows supplemented with HMTBa had 35.3% higher *trans*-11 C18:1, 2.3-fold lower *trans*-10 C18:1, and higher de novo FA (<16 C) concentration in milk fat (Figures 2 and 3). Individual FA <16 carbons generally followed a similar pattern (Supplemental Tables S3, S4, and S5; <https://doi.org/10.3168/jds.2017-13446>). Although a treatment by

day interaction was not observed for OBCFA, in high-producing cows during the high-risk phase HMTBa increased C15:0 and C17:0 (Supplemental Figure S1, <https://doi.org/10.3168/jds.2017-13446>), and resulted in greater total OBCFA concentration (3.04 vs. 2.79 g/100 g of total FA, $P = 0.04$; Supplemental Table S2).

DISCUSSION

The sequentially increasing risk factor design allowed the efficient investigation of the interaction between HMTBa, susceptibility to MFD due to production level, and dietary challenge (low, moderate, and high risk for BH-induced MFD). Decreasing diet NDF and increasing starch and UFA is a well-characterized model to alter ruminal BH pathways and induce the formation of bioactive FA that inhibit milk fat synthesis (Bauman and Griinari, 2003; Rico and Harvatine, 2013; Rico et al., 2014a). Increasing starch concentration from 24.4 to 26.8 to 29.2% of the diet is expected to have decreased rumen pH and altered microbial populations to those adaptable to starch digestion and lower pH, although neither were measured in the current experiment. The physically effective NDF during the moderate- and high-risk diets (17.3 and 15.1%DM, respectively) was below the suggested threshold of 21% required to maintain rumen pH below 6.0 (Zebeli et al., 2007). Of note, responses to HMTBa have been demonstrated to be independent of rumen pH (Vázquez-Añón et al., 2001; Lee et al., 2015). Increasing diet PUFA from 2.2 to 2.7 and 3.1% of the diet increased the amount of rumen-available UFA requiring BH and is also expected to have modified the microbial population by selecting against populations sensitive to UFA.

Rico and Harvatine (2013) characterized the time-course of BH-induced MFD and demonstrated that milk fat content and yield were decreased by d 3 and 5, respectively, and the maximal response occurred in approximately 7 to 10 d. Additionally, concentration of *trans*-10 C18:1 and *trans*-10,*cis*-12 CLA in milk fat increased progressively and were higher than control on d 1 and 3, respectively. Changes in key microbial populations have also been demonstrated to occur over a similar rapid time-course during BH-induced MFD (Rico et al., 2015). In summary, we highlighted the fast adaptation of rumen microbes to changes in diet fermentability and UFA and supported the use of 10- to 14-d experimental periods to investigate BH-induced MFD.

Cows were grouped based on level of milk production, but DIM, DMI, and milk fat and protein concentrations and yields also differed between the blocks, as expected. A 3-way interaction of treatment, dietary risk, and production level for milk fat concentration

Figure 1. Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) supplementation (0.1% of DM) on milk fat concentration (A) and yield (B) in high- (Con-H and HMTBa-H) and low-producing (Con-L and HMTBa-L) cows receiving diets with low, moderate, and high risk for biohydrogenation-induced milk fat depression. High-producing cows averaged 44.1 ± 4.5 kg/d and low-producing cows averaged 31.4 ± 4.3 kg/d. Effect of treatment (control vs. HMTBa) within each dietary phase in high-producing cows is shown (* $P < 0.05$ and ** $P < 0.01$). Treatments (Trt) did not differ at any time point in low-producing cows ($P > 0.05$). Pooled SEM for milk fat concentration was 0.22 and 0.20 in low- and high-producing cows, respectively. Pooled SEM for fat yield was 0.12 and 0.09 in low- and high-producing cows, respectively.

Table 5. Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) on selected milk fatty acids (FA) in high- and low-producing cows

FA (g/100 g of total FA)	Block ¹	Treatment ²			P-values ³		
		Control	HMTBa	SEM	Trt	Day	Trt × day
<i>trans</i> -11 C18:1	H	1.34	1.56	0.10	0.16	<0.001	<0.001
	L	1.60	1.90	0.16	0.23	<0.001	0.15
<i>trans</i> -10 C18:1	H	2.46	1.00	0.34	0.01	<0.001	<0.001
	L	0.66	0.66	0.03	0.99	<0.001	0.50
Σ <16 C ⁴	H	21.9	22.3	1.11	0.80	<0.001	0.01
	L	23.4	23.4	0.30	0.96	<0.001	0.88
Σ 16 C ⁴	H	25.6	24.3	1.65	0.59	0.05	0.39
	L	24.5	23.9	0.38	0.30	<0.001	0.17
Σ >16 C ⁴	H	41.8	40.3	1.54	0.53	<0.001	0.58
	L	44.6	45.2	0.57	0.49	<0.001	0.48
Σ OBCFA ⁵	H	3.34	3.44	0.04	0.17	<0.0001	0.11
	L	3.42	3.43	0.03	0.74	<0.0001	0.97

¹Milk production level: high-producing cows (H) averaged 44.1 ± 4.5 kg/d and low-producing cows (L) averaged 31.4 ± 4.3 kg/d at the end of the pretrial period.

²Treatment was control (ground corn) or HMTBa (0.1% of diet DM provided in a corn carrier).

³Effect of treatment (Trt), experimental day, and their interaction.

⁴FA <16 C originate from de novo synthesis in the mammary gland, FA >16 C originate from extraction from plasma, and 16 C FA originate from both sources.

⁵Sum of all odd- and branched-chain fatty acids in milk.

required the analysis within production blocks. Briefly, BH-induced MFD did not occur in low-producing cows, and the effects of HMTBa supplementation would have to occur through a different mechanism to affect milk fat production in this block. The low-producing group was composed primarily by late-lactation cows. Energy status of the cow may be a more important determinant of hormonal profile than stage of lactation (Peel et al., 1983), and cows in the high-producing group were mid-lactation and also expected to be in a positive energy balance. We did not observe any treatment by production level interaction for plasma variables. Therefore, the differential response of low- and high-producing cows to dietary BH-induced MFD risk factors and HMTBa supplementation in the current study was not expected to have been mediated by hormonal status. Furthermore, as reviewed by Harvatine et al. (2009a), *trans*-10,*cis*-12 CLA (a potent bioactive *trans* FA formed during altered ruminal BH) reduces milk fat yield during all phases of the lactation cycle. This indicates that, in the current study, the absence of MFD in low-producing cows was not related to a lower responsiveness of the mammary gland to bioactive *trans* FA that inhibit milk fat synthesis and is likely due to a reduced flow of bioactive FA from the rumen.

The high-producing cows in the current experiment had higher DMI. Jenkins et al. (2008) proposed that the kinetics of ruminal passage of feed particles directly affects the rates of lipolysis and BH. Rumen passage rate is expected to increase as DMI increases, which may modify the microbial population and increase ruminal outflow of *trans* intermediate FA before complete BH has occurred. Additionally, high-producing cows

may differ in feeding and ruminating behavior (e.g., increased meal size, slug feeding), which may result in subclinical rumen acidosis and further alteration of normal microbial populations and BH pathways (Harvatine, 2017). Milk *trans*-10 C18:1, a proxy of altered ruminal BH, was 38% higher in high-producing cows at the end of the pretrial period, indicating presence of the alternate ruminal BH pathway before initiation of the trial. Furthermore, it suggests that high-producing cows may be at higher risk of BH-induced MFD even under a low-risk diet, which has been reported in 2 other studies with similar high- and low-producing blocks (Harvatine and Allen, 2006; Rico et al., 2014b).

Increasing dietary risk factors resulted in pronounced BH-induced MFD in high-producing cows in the control treatment, as evidenced by a 14 and 30% decrease in milk fat concentration during the moderate- and high-risk phases, respectively. The extent of MFD in the high-risk phase is within the range of 25 to 38% observed using similar changes in diet fermentability and UFA content (Peterson et al., 2003; Harvatine and Bauman, 2006; Rico and Harvatine, 2013). The phenotype of the response observed in the current experiment, including decreased milk fat with no changes in milk yield or yield of other components, is characteristic of classical BH-induced MFD (Harvatine et al., 2009b). This is further supported by the substantial 7.9-fold increase in *trans*-10 C18:1 in milk fat of high-producing cows in the control treatment. *Trans*-10 C18:1 is a major intermediate formed during altered BH (Jenkins et al., 2008), and the concentration of *trans*-10 C18:1 in milk can serve as a proxy to characterize the condition. This is mostly because absorbed FA are rapidly incor-

porated into milk fat (Harvatine and Bauman, 2011) and because the majority (>85%) of preformed milk FA come from absorption in cows in a positive energy balance (Palmquist and Mattos, 1978).

We observed a 3-way interaction of treatment, dietary phase, and production level, where HMTBa supplementation resulted in greater milk fat concentration during

Figure 2. Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) supplementation (0.1% DM) on the milk fat concentration of the predominant isomers of the normal (*trans*-11 C18:1; A) and the alternate (*trans*-10 C18:1; B) biohydrogenation pathways in high- (Con-H and HMTBa-H) and low-producing (Con-L and HMTBa-L) cows receiving diets with low, moderate, and high risk for biohydrogenation-induced milk fat depression. High-producing cows averaged 44.1 ± 4.5 kg/d and low-producing cows averaged 31.4 ± 4.3 kg/d. The treatment (Trt) by day interaction for high- (H) and low-producing (L) cows is shown. Significant differences between control and HMTBa in high-producing cows within each dietary phase are shown (*** $P < 0.01$). Treatments did not differ ($P > 0.05$) at any time point in low-producing cows. Pooled SEM for *trans*-11 C18:1 was 0.40 and 0.20 in low- and high-producing cows, respectively. Pooled SEM for *trans*-10 C18:1 was 0.06 and 0.73 in low- and high-producing cows, respectively. FA = fatty acid.

Figure 3. Effect of 2-hydroxy-4-(methylthio)butanoate (HMTBa) supplementation (0.1% diet DM) on milk fatty acid (FA) concentration by source in high-producing cows (44.1 ± 4.5 kg/d) receiving diets with low, moderate, and high risk for biohydrogenation-induced milk fat depression. Fatty acids <16 C originate from de novo synthesis in the mammary gland (A), FA >16 C are extracted from plasma (C), and 16-C FA from both sources (B). Significance (* $P < 0.05$) is presented for the control versus HMTBa within each dietary phase.

the moderate- and high-risk phases and greater milk fat yield in the high-risk phase in high-producing cows. Greater milk fat synthesis in HMTBa-supplemented high-producing cows resulted primarily from maintenance of the de novo FA synthesis pathway in the mammary gland. Supplementation with HMTBa, which is a hydroxyl analog of methionine, was initially intended to provide additional metabolizable methionine to the cow. However, with a reported ruminal degradability of 50% or more (Koenig et al., 1999; Noftsger et al., 2005), HMTBa may also affect rumen microbial composition or growth by providing methionine or through a rumen modifier mechanism. In fact, HMTBa has been suggested to increase bacterial N synthesis in vitro and in vivo, which could be associated with increased microbial protein yield (Vázquez-Añón et al., 2001; Lee et

al., 2015). Early work proposed that the positive effect of HMTBa on milk fat was due to a direct effect of HMTBa on rumen lipid metabolism (Patton et al., 1970). However, the effect of HMTBa on milk fat is variable, but more apparent when feeding diets with increased risk of MFD. For example, Rosser et al. (1971) reported that supplementation with a methionine analog maintained milk fat synthesis when cows were fed a highly fermentable diet (85% pelleted concentrate). On the contrary, no increase in milk fat was observed when low-risk diets (>33% NDF and <3% ether extract) were supplemented with HMTBa (Johnson-VanWieringen et al., 2007; Lee et al., 2015).

In high-producing cows, HMTBa supplementation during the high-risk phase decreased the concentration of alternate BH isomers (*trans*-10 C18:1) and increased isomers of the normal pathway (*trans*-11 C18:1). This indicates a role for HMTBa in stabilizing rumen BH and reducing the shift to the altered *trans*-10 pathways that occurs during BH-induced MFD. This positive effect of HMTBa on ruminal BH could be mediated through 2 mechanisms. First, if an increase in microbial mass occurs with HMTBa supplementation (see discussion above), an increase in BH capacity would be expected. Second, HMTBa may modify the microbial population and maintain key ruminal microbial species important to BH. Rico et al. (2015) characterized the shift in common culturable microbial populations during BH-induced MFD and reported an increase in the abundance of *Streptococcus bovis* (amylolytic) and *Megasphaera elsdenii* and *Selenomonas ruminantium* (lactate-utilizing bacteria) and a decrease in *Fibrobacter succinogenes* (fibrolytic) and *Butyrivibrio fibrisolvens*/*Pseudobutyrvibrio* group (BH-involved). Several studies have indicated that bacteria other than *Butyrivibrio* spp. might be involved in BH (Shingfield and Wallace, 2014), but the key populations in vivo are not clear. Additionally, Martin et al. (2013) observed an increase in the abundance of *Fibrobacter succinogenes* in rumen contents of cows supplemented with a methionine analog. Although *F. succinogenes* has been described primarily as fibrolytic, a role for this species in the BH of linoleic acid (C18:2) has also been proposed (Shingfield and Wallace, 2014). Baldin et al. (2015) evaluated the effect of HMTBa on microbial populations when feeding diets with increased risk of MFD. Even though there was an increase in the abundance of rumen protozoa, those authors reported no changes in 9 selected microbial taxa with well-characterized functions, including 2 species (*Butyrivibrio*/*Pseudobutyrvibrio*, *Butyrivibrio hungatei*) involved in *trans*-11 production. Of note, the bacterial species measured in the study of Baldin et al. (2015) represented only a small fraction of the total rumen microbiome that can be potentially important to

ruminal BH. Characterization of microbial population was beyond the scope of the current experiment.

A postrumen effect of methionine and analog compounds on milk fat synthesis has also been proposed (Patton et al., 1970; Huber et al., 1984; Zanton et al., 2014). Methionine plays an important role in choline synthesis, and methionine and choline both act as methyl donors, thereby potentially influencing milk volume and milk fat (Zanton et al., 2014). Additionally, methionine is involved in the transport of lipids (Patton et al., 1970) and its supplementation has been shown to increase blood triglycerides (Huber et al., 1984), which are a key substrate for milk fat synthesis. Contributions of postrumen effects to the milk fat response observed in the current study cannot be ruled out, but we expect that the rumen mechanism played the predominant role.

The experimental design used in our study verified the existence of interactions between dietary risk factors for MFD, animal characteristics, and a feed additive. Our approach provided an efficient way to investigate these interactions and demonstrated the potential of HMTBa to reduce BH-induced MFD. This important interaction could not have not been identified if the study had looked at a single factor at a time and should also represent on farm scenarios where gradual modifications in the diet increase the risk of BH-induced MFD.

CONCLUSIONS

High-producing cows had lower milk fat concentration than low-producing cows. During the treatment period, increasing diet UFA and carbohydrate fermentability caused MFD in high-producing cows receiving the control treatment, but HMTBa supplementation maintained higher milk fat concentration and yield, especially during the high-risk phase. Additionally, HMTBa maintained lower concentration of *trans*-10 C18:1 in milk; which suggested a role for HMTBa in stabilizing rumen BH and preventing the shift to the altered BH pathway. Low-producing cows neither experienced substantial BH-induced MFD nor a response in milk fat to HMTBa supplementation. Characterization of the interaction of dietary risk factors, milk production level, and HMTBa supplementation provides a mechanism to prevent or attenuate BH-induced MFD on farms and reduces its occurrence in high-producing cows within herds with normal milk fat. The experimental model offers a framework to further study other rumen modifiers that can combat BH-induced MFD.

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