Effects of diet fermentability and supplementation of 2-hydroxy-4-(methylthio)-butanoic acid and isoacids on milk fat depression: 1. Production, milk fatty acid profile, and nutrient digestibility

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ABSTRACT

The objectives of this experiment were to determine the effects of increased diet fermentability and polyunsaturated fatty acids (FA) with or without supplemental 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa), isoacids (IA; isobutyrate, 2-methylbutyrate, isovalerate, and valerate) or the combination of these on milk fat depression (MFD). Ten Holstein cows (194 ± 58 DIM, 691 ± 69 kg BW, 28 ± 5 kg milk yield) were used in a replicated 5×5 Latin square design. Treatments included a high-forage control diet (HF-C), a low-forage control diet (LF-C) causing MFD by increasing starch and decreasing neutral detergent fiber (NDF), the LF-C diet supplemented with HMTBa at 0.11% (28 g/d), the LF-C diet supplemented with IA at 0.24% of dietary dry matter (60 g/d), and the LF-C diet supplemented with HMTBa and IA. Preplanned contrasts were used to compare HF-C versus LF-C and to examine the main effects of HMTBa or IA and their interactions within the LF diets. Dry matter intake was greater for LF-C versus HF-C, but milk yield remained unchanged. The LF-C diet decreased milk fat yield (0.87 vs. 0.98 kg/d) but increased protein yield compared with HF-C. As a result, energy-corrected milk was lower (28.5 vs. 29.6 kg/d) for LF-C versus HF-C. Although the concentration of total de novo synthesized FA in milk fat was not affected, some short- and medium-chain FA were lower for LF-C versus HF-C, but the concentrations of C18 *trans*-10 isomers were not different. Total-tract NDF apparent digestibility was numerically lower (42.4 vs. 45.6%) for LF-C versus HF-C. As the main effects, the decrease in milk fat yield observed in LF-C was alleviated by supplementation of HMTBa through increasing milk yield without altering milk fat content and by IA through increasing milk fat content without altering

milk yield so that HMTBa or IA, as the main effects, increased milk fat yield within the LF diets. However, interactions for milk fat yield and ECM were observed between HMTBa and IA, suggesting no additive effect when used in combination. Minimal changes were found on milk FA profile when HMTBa was provided. However, de novo synthesized FA increased for IA supplementation. We detected no main effect of HMTBa, IA, and interaction between those on total-tract NDF digestibility. In conclusion, the addition of HMTBa and IA to a low-forage and high-starch diet alleviated moderate MFD. Although the mechanism by which MFD was alleviated was different between HMTBa and IA, no additive effects of the combination were observed on milk fat yield and ECM.

Key words: low-forage diet, methionine analog, branched-chain volatile fatty acids, valerate

INTRODUCTION

Milk fat depression (MFD) that is induced by dietary changes has been studied for decades, and among various theories proposed and studied, biohydrogenation-induced MFD, also called classical diet-induced MFD, has successfully explained MFD to some extent (Jenkins and Harvatine, 2014; Dewanckele et al., 2020). Biohydrogenation-induced MFD occurs with a shift of *trans*-11 to *trans*-10 C18 fatty acids (FA)—that is, alternate biohydrogenation pathway—directly inhibiting milk fat synthesis in mammary glands. This alternate biohydrogenation is enhanced when diet fermentability and PUFA content increase (Fuentes et al., 2009; Jenkins and Harvatine, 2014), causing severe MFD (Rico and Harvatine, 2013; Rico et al., 2015). However, changes in milk *trans*-10 C18 isomers do not fully explain MFD, indicating the existence of unknown other factors, and some cases of MFD have not been exactly characterized as the biohydrogenation-induced MFD (Dewanckele et al., 2020). Moderate MFD has received less attention and could be one type of MFD that may

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be poorly explained by the biohydrogenation theory (Baldin et al., 2018).

2-Hydroxy-4-(methylthio)-butanoic acid (**HMTBa**) was originally designed as a methionine precursor to support milk protein synthesis (Lobley et al., 2006), but meta-analyses indicated that HMTBa often increased milk fat yield (Zanton et al., 2014; Feng et al., 2018). This effect of HMTBa is different from the effect of rumen-protected methionine or D- or L-methionine infusion into the abomasum, the major effect of which was an increase in milk protein yield (Zanton et al., 2014). Recently, supplementing MFD-inducing diets with HMTBa has been shown to alleviate risk of MFD in high-producing cows (Baldin et al., 2018; Pitta et al., 2020).

Branched-chain VFA (isobutyric, isovaleric, and 2-methylbutyric; **BCVFA**) and valerate (a straight-chain acid) are commonly referred to as isoacids (**IA**) produced during feed fermentation in the rumen and are growth factors especially for cellulolytic bacteria (Allison et al., 1962a,b; Dehority et al., 1967). Supplementation of IA (either individual BCVFA, all BCVFA, or IA) have often been shown to increase milk fat, milk yield, and fiber degradability in vitro and in vivo (Rogers et al., 1989; Yang, 2002; Wang et al., 2012). During in vitro studies by Kone et al. (1989) and Yang (2002), IA supply increased acetate and total VFA production, and increased NDF digestibility was also observed. This could be an evidence of IA stimulating milk fat synthesis because acetate supplementation can increase milk fat synthesis (Urrutia et al., 2019). Previous in vivo studies (Papas et al., 1984; Peirce-Sandner et al., 1985; Liu et al., 2018; Wang et al., 2019) also showed that supplemental IA increases fiber digestion or milk fat yield in dairy cows and could be effective in alleviating MFD.

We expected that increased diet fermentability with PUFA supplementation would cause moderate MFD in low-producing cows, resulting from alternate biohydrogenation pathways. Supplementing an MFD-inducing diet with HMTBa and IA was hypothesized to reduce risk of MFD, and the combination of the 2 supplements was predicted to have an additive effect. The objective of this study was to determine milk production, FA profile, and nutrient digestibility in lactating cows fed MFD-inducing diets supplemented with HMTBa and IA to test these hypotheses.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

The experiment was performed at Krauss Dairy Research Center at Agricultural Research and Devel-

opment Center, The Ohio State University (Wooster, OH). All procedures involving animals and their care were approved (2018A00000116) by the Institutional Animal Care and Use Committee of The Ohio State University.

A total of 10 multiparous lactating Holstein cows (mean $\pm$ SD: 194 $\pm$ 58 DIM, 691 $\pm$ 69 kg BW, and 28 $\pm$ 5 kg/d milk yield) were used in a replicated 5 $\times$ 5 Latin square design (balanced) and housed in individual tie stalls. Square 1 included 5 noncannulated cows, and square 2 included 5 cannulated cows that were used to evaluate ruminal fermentation characteristics, FA profile, and bacterial community data, which were presented in our companion paper (Lee et al., 2021). The experiment consisted of 5 periods with 21 d per period (14 d for adaption and 7 d for sampling). As shown in Table 1, dietary treatments included a relatively high-forage and low-starch control diet with 1.5% safflower oil (**HF-C**); a relatively low-forage and high-starch control diet with 1.5% safflower oil (**LF-C**); the LF-C diet supplemented with 0.11% of HMTBa in dietary DM (28 g/d; Adisseo, Antony, France; **LF-HMTBa**); the LF-C diet supplemented with 0.24% of IA in dietary DM (Zinpro Corp., Eden Prairie, MN), supplying about 60 g/d of IA (17 g/d of isobutyrate, 17 g/d of 2-methylbutyrate, 12 g/d of isovalerate, and 14 g/d of valerate; **LF-IA**); and LF-C plus the combination of HMTBa and IA (**LF-COMB**). All diets included safflower oil (about 70% linoleic acid content; Jedwards International Inc., Braintree, MA) to provide linoleic acid (about 37% linoleic acid of total dietary FA). Diets were formulated to meet or exceed the requirement of all nutrients for cows of 24 kg/d DMI, 35 kg/d milk yield, and 700 kg BW, according to NRC (2001). The concentrates were prepared for each diet, mixed daily with forages as a TMR, and fed to cows for ad libitum intake with orts targeted at about 5%. Cows had free access to water and were milked twice daily at 0400 and 1600 h.

Sampling Procedure

Forages were sampled weekly and dried at 100°C overnight, and DM of forages were used to adjust diets weekly. Concentrates and forages were sampled once a week, and orts from individual cows were sampled on d 19 to 21 in each period. Feed samples were composited by period, and orts samples were composited by cow within period for chemical analyses later.

Spot fecal and urine samples were collected from individual cows (total 6 spot samples per cow; about 300 g of sample at each event) on d 18 to 21 in each period to represent every 4-h sampling in a 24-h cycle (Morris

et al., 2018; Lee et al., 2019). Fecal samples collected at each time point were composited on an equal wet-weight basis by cow and period. Each spot urine sample collected through vulva stimulation was acidified with 2 M H₂SO₄ and composited on an equal-weight basis by cow and period.

Samples of milk were taken weekly and milk composition determined (milk fat, true protein, lactose, and MUN; B2000 infrared analyzer, Bentley Instruments, Chaska, MN) by DHI Cooperative Inc. (Columbus, OH). Additional milk samples during a.m. and p.m. milking were collected on d 16 and 17, composited, and centrifuged at 17,800 × *g* for 30 min at 4°C; the fat cake was removed and frozen at −20°C for determination of FA profile. Another set of milk samples was collected during a.m. and p.m. milkings on d 18 to 20 and immediately analyzed for total N.

Laboratory Analyses

Composite feed ingredients, refusals, and fecal samples were dried at 55°C for 72 h and ground to pass a 1-mm screen (Wiley mill; Arthur H. Thomas Co., Philadelphia, PA). Dried and ground fecal samples were analyzed for DM at 100°C (method 934.01; AOAC International, 2000), ash (method 942.05; AOAC International, 2000), N (Morris et al., 2019), and NDF (amylase, sodium sulfite; Ankom200 Fiber Analyzer; Ankom Technology Corp., Fairport, NY). In addition, feeds, refusals, and feces were analyzed for indigestible NDF as described by Huhtanen et al. (1994), with the following modification. Samples were weighed in quadruplicate into filter bags (25-μm pore size; Ankom Technology Corp.) and incubated in 2 cannulated lactating Holstein cows (about 60% forage and 40%

Table 1. Ingredient and chemical composition of diets

Item	Diet ¹				
	LF				
	HF-C	LF-C	LF-HMTBa	LF-IA	LF-COMB
Ingredient, % of DM					
Corn silage	38.1	36.8	36.8	36.8	36.8
Alfalfa silage	18.3	11.7	11.7	11.7	11.7
Corn grain, ground	12.0	20.0	19.9	20.0	19.9
Soybean meal	13.9	15.8	15.8	15.8	15.8
Soybean hulls	13.7	11.9	11.9	11.7	11.7
Safflower oil ²	1.49	1.49	1.49	1.49	1.49
Vitamin and mineral mix ³	2.68	2.68	2.68	2.68	2.68
HMTBa ⁴	—	—	0.11	—	0.11
Isoacids ⁵	—	—	—	0.24	0.24
Chemical composition, % of DM					
OM	93.3	93.7	93.8	93.6	93.5
CP	15.8	15.9	15.7	15.9	15.9
NDF	32.2	28.9	28.9	28.8	28.3
Forage NDF	20.1	17.4	17.4	17.4	17.4
Starch	24.3	29.6	29.1	29.3	29.4
FA ⁶	3.4	3.5	3.7	3.7	3.5
C8:0	1.32	1.33	1.29	1.27	1.22
C16:0	14.8	14.5	14.0	14.0	14.4
C16:1	0.88	0.83	0.86	0.84	0.86
C18:0	1.89	1.64	1.56	1.57	1.72
C18:1 <i>cis</i> -9	9.63	9.35	9.34	9.36	9.56
C18:1 <i>cis</i> -11	0.69	0.61	0.59	0.58	0.60
C18:2 <i>cis</i> -9, <i>cis</i> -12	34.4	36.2	38.5	38.6	37.0
C18:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12	6.28	6.29	6.35	6.37	6.34

¹HF-C = high-forage and low-starch diet; LF = low-forage and high-starch diets; LF-C = low-forage and high-starch diet with no supplementation; LF-HMTBa = LF-C diet supplemented with 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa); LF-IA = LF-C diet supplemented with isoacids (IA); LF-COMB = LF-C diet supplemented with HMTBa and IA.

²Jedwards International Inc., Raintree, MA.

³Contained 38% trace mineral premix (39.3% sodium, 53.2% chloride, 70 mg/kg cobalt, 400 mg/kg copper, 70 mg/kg iodine, 1,750 mg/kg iron, 2,800 mg/kg manganese, 200 mg/kg Se, and 3,500 mg/kg zinc), 30% limestone, 24% dicalcium phosphate, 0.55% vitamin A (30 kIU/g), 1.6% vitamin D (3 kIU/g), and 2.0% vitamin E (44 IU/g).

⁴2-Hydroxy-4-(methylthio)-butanoic acid (Rhodiment, Adisseo, Antony, France).

⁵Contained 7.3% isobutyrate, 7.2% 2-methylbutyrate, 4.9% isovalerate, and 5.9% valerate (Zinpro Corp., Eden Prairie, MN).

⁶FA = fatty acid, % of total fatty acids.

concentrate; similar to HF-C without safflower oil) for 12 d. After incubation, bags were rinsed with cold water and analyzed for NDF. Total fecal output was estimated using indigestible NDF intake and fecal indigestible NDF content. Urine samples were analyzed for total N (Morris et al., 2019), creatinine (Cayman Chemical, Ann Arbor, MI), and urea N (Kit UR107; Randox Laboratories Ltd., Crumlin, UK). Daily urine excretion was estimated based on urinary creatinine concentration using creatinine excretion rate of 29.0 mg/kg of BW (Valadares et al., 1999; Tebbe and Weiss, 2018). Milk samples were analyzed for total N (Morris et al., 2019). Milk FA profile was determined using the methylation procedure by Jenkins (2010), with separation by gas chromatography (HP 5890 series, Agilent Technologies, Santa Clara, CA) using a CP-SIL88 capillary column (100 m $\times$ 0.25 mm $\times$ 0.2- μ m film thickness; Varian Inc., Palo Alto, CA). Individual FA were quantified based on the areas after normalizing the internal standard (C19:0), but recovery factors for individual FA were not determined and adjusted (for example, see Urrutia and Harvatine, 2017). We also noticed that the methylation method used here may have caused losses of shorter-chain FA (especially C4:0 and C6:0), although those FA were not likely lost, based on their concentrations in milk compared with those from the sodium methoxide methylation in the literature (Urrutia and Harvatine, 2017).

Statistical Analysis

Data from 1 cannulated cow in period 3 on the LF treatment were removed from statistical analysis due to mastitis. This cow recovered quickly and completed period 4. All cows (cannulated and noncannulated) were used to obtain data for nutrient digestibility, milk production, and milk FA profiles.

Milk yield, DMI, and feed efficiency data collected during the last 7 d of each period were used for statistical analysis using the MIXED procedure of SAS (SAS 9.4; SAS Institute Inc., Cary, NC). The mixed model included the fixed effects of treatment, period, day, and treatment by day interaction; it also included the random effects of square and cow within square. Day was used for repeated measures with the covariance structure of autoregressive order (1) according to the lowest Akaike information criterion. Degrees of freedom were adjusted using the option of Kenward-Roger. Data of milk composition, nutrient digestibility, and milk fatty acid profiles were analyzed using the same model, except that repeated measures were not included.

Preplanned contrasts were used to determine the difference between HF-C and LF-C. The main effect

of HMTBa (LF-HMTBa and LF-COMB vs. LF and LF-IA), IA (LF-IA and LF-COMB vs. LF and LF-HMTBa), and their interaction were tested. Therefore, effects of HMTBa or IA are referred to as the main effects of HMTBa or IA, respectively, in the Results and Discussion sections. When an interaction was $P \leq 0.10$, the PDIFF option generated pairwise comparisons among treatments (i.e., Fisher's protected least significant difference). Statistical differences were declared at $P \leq 0.05$. Differences between treatments with $0.05 < P \leq 0.10$ were considered as trends toward significance.

RESULTS

Dry Matter Intake, Milk Production, and Milk Composition

The LF-C diet increased (25.3 vs. 24.8 kg/d; $P = 0.04$) DMI compared with HF-C (Table 2), but milk yield was not affected. Milk fat content ($P = 0.03$) and milk fat yield (0.87 vs. 0.98 kg/d; $P < 0.01$) decreased for LF-C versus HF-C, resulting in decreases in ECM ($P = 0.03$) for LF-C versus HF-C. Milk protein concentration and yield were greater ($P \leq 0.01$) for LF-C compared with HF-C. The main effects of HMTBa or IA had no effect on DMI, but a tendency ($P = 0.08$) for an interaction existed between HMTBa and IA. Milk yield tended to increase (30.1 vs. 29.4 kg/d; $P = 0.06$) for HMTBa but was not affected by IA within the LF diets. An interaction ($P = 0.01$) was found between HMTBa and IA for milk yield. We detected no main effect of HMTBa on milk protein content and yield, but IA decreased (1.01 vs. 1.04 kg/d; $P = 0.04$) milk protein yield within the LF diets without an interaction between HMTBa and IA. Supplementation of HMTBa did not affect milk fat content but increased ($P < 0.01$) milk fat yield. Although milk fat content was not affected by IA, milk fat yield increased ($P < 0.01$) for IA within the LF diets. An interaction ($P < 0.01$) for milk fat yield between HMTBa and IA was detected. The main effects of HMTBa and IA were not significant for lactose yield, but HMTBa and IA interacted ($P < 0.01$). No difference in MUN was observed among treatments.

Milk Fatty Acid Concentrations and Yield

Changes in milk FA profile were observed by treatments (Table 3). For LF-C, the concentration of C6:0 and C8:0 decreased ($P = 0.01$), and that of C14:0 tended to decrease ($P = 0.09$), but total de novo synthesized FA showed only numerical decreases ($P = 0.18$) compared with HF-C. The concentration of linoleic acid

Table 2. Effects of supplementing a diet inducing milk fat depression with 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa) and isoacids (IA) on intake and milk production and composition of lactating cows (n = 10)

Item	Diet ¹					SEM	<i>P</i> -value ²			
	HF-C	LF					CON	HMTBa	IA	INT
		LF-C	LF-HMTBa	LF-IA	LF-COMB					
BW, kg	755	759	763	768	762	26.1	0.47	0.80	0.28	0.16
BW change, kg/d	1.1	1.0	0.90	1.0	1.0	0.25	0.69	0.75	0.67	0.84
DMI, kg/d	24.8	25.3	25.8	25.9	25.7	1.05	0.04	0.79	0.29	0.08
Milk, kg/d	28.9	29.0	30.6	29.8	29.5	5.28	0.86	0.06	0.60	0.01
ECM, ³ kg/d	29.6	28.5	31.0	30.6	29.7	6.04	0.03	0.03	0.30	<0.01
ECM/DMI, kg/kg	1.19	1.12	1.20	1.17	1.15	0.20	<0.01	0.07	0.78	<0.01
Milk composition										
Fat, %	3.38	2.97	3.27	3.41	3.23	0.20	0.03	0.63	0.13	0.09
True protein, %	3.37	3.51	3.41	3.40	3.45	0.09	0.04	0.56	0.43	0.07
Lactose, %	4.74	4.79	4.78	4.77	4.78	0.09	0.39	0.82	0.86	0.59
Fat, kg/d	0.98	0.87	1.00	1.01	0.96	0.22	<0.01	0.02	<0.01	<0.01
Protein, kg/d	0.98	1.02	1.05	1.01	1.01	0.18	0.01	0.26	0.04	0.46
Lactose, kg/d	1.38	1.41	1.48	1.44	1.41	0.27	0.20	0.23	0.21	<0.01
MUN, mg/dL	12.9	12.6	12.7	13.8	13.0	0.65	0.72	0.50	0.14	0.39

¹HF-C = high-forage and low-starch diet; LF = low-forage and high-starch diet; LF-C = low-forage and high-starch diet with no supplementation; LF-HMTBa = LF-C diet supplemented with HMTBa; LF-IA = LF-C diet supplemented with IA; LF-COMB = LF-C diet supplemented with HMTBa and IA.

²CON = HF-C vs. LF-C; HMTBa = main effect of HMTBa in the LF diets; IA = main effect of IA in the LF diets; INT = interaction of HMTBa with IA.

³ECM (kg/d) = kg of milk × [(38.3 × % fat × 10 + 24.2 × % true protein × 10 + 16.54 × % lactose × 10 + 20.7)/3,140] (Sjaunja et al., 1990).

(18:2 *cis*-9, *cis*-12) was greater (4.48 vs. 3.80% of total FA; $P < 0.01$) for LF-C versus HF-C. As the main effect, HMTBa had no effect on milk FA profile within the LF diets, including C18 *trans*-10 or 11 isomers. As the main effect, IA supplementation increased (29.3 vs. 27.4% of total FA; $P = 0.01$) the concentration of de novo synthesized FA (C6:0, C8:0, C10:0, C12:0, C14:0, and C15:0) and decreased ($P = 0.03$) that of preformed FA (C17:0 iso, linoleic acid, and various *cis* or *trans* C18:1) within the LF diets. In addition, IA decreased ($P = 0.04$) *trans*-11 C18:1 and tended to decrease *cis*-9, *trans*-11 C18:1 within the LF diets. A tendency of interactions ($P \leq 0.10$) between HMTBa and IA for minor FA was observed.

Nitrogen Balance and Total-Tract Apparent Nutrient Digestibility

No major differences in dietary N utilization for milk N and excretion in feces and urine were detected among treatments (Table 4) except those between LF-C and HF-C. The LF-C diet had greater ($P = 0.01$) N intake and tended to have lower (35.1 vs. 38.1%; $P = 0.06$) fecal and (30.9 vs. 33.2%; $P = 0.05$) urinary N excretion as percentages of N intake compared with HF-C. This resulted in an increase in the amount of N retained (58.5 vs. 18.8 g/d; $P = 0.01$) and retained N as proportion of N intake (8.6 vs. 2.9%; $P = 0.05$) for LF-C com-

pared with HF-C. However, no main effects of HMTBa or IA and their interaction was observed on dietary N utilization and excretion, which suggests that all of the LF diets supported greater N retention compared with cows fed the HF-C diet. Apparent total-tract nutrient digestibility did not differ among treatments. However, compared with HF-C, LF-C numerically decreased (42 vs. 46%; $P = 0.12$) NDF digestibility (Table 5), and neither HMTBa nor IA had significant main effects. No interactions for nutrient digestibility were observed.

DISCUSSION

In the current study, the MFD-inducing control diet (i.e., LF-C) was obtained by lowering NDF (32 to 29%; forage NDF from 20 to 17% on a DM basis) and increasing starch concentration from 24 to 29% in dietary DM compared with HF-C. Safflower oil (1.5% of dietary DM) was also included in LF-C as a source of PUFA (mainly linoleic acid). Safflower oil was included in the HF-C diet at the same inclusion rate as in LF-C, to examine whether or not alternate biohydrogenation is enhanced under LF-C versus HF-C. The inclusion rate of isobutyrate, 2-methylbutyrate, isovalerate, and valerate for IA was based on previous studies by Papas et al. (1984) and Liu et al. (2018). Liu et al. (2018) recommended that equimolar proportions of BCVFA at total 60 g per day (excluding valerate) was the optimal,

based on a dose response of milk production to BCFVA. The inclusion rate of 0.1% HMTBa in dietary DM was used according to previous studies (Baldin et al., 2018; Pitta et al., 2020) in which MFD was alleviated by supplementing MFD-inducing diets with HMTBa.

Effects of LF-C Versus HF-C

Cows fed LF-C had moderate MFD, as designed, compared with those fed HF-C. The degrees of the decreases in milk fat content and yield were only 12 and

Table 3. Effects of supplementing a diet inducing milk fat depression with 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa) and isoacids (IA) on milk fatty acid profile of lactating cows (n = 10)

Fatty acid, g/100 g	Diet ¹						P-value ²			
	LF					SEM				
	HF-C	LF-C	LF-HMTBa	LF-IA	LF-COMB		CON	HMTBa	IA	INT
4:0	5.36	5.50	4.90	5.48	5.49	0.415	0.79	0.23	0.29	0.39
6:0	2.33	1.91	1.97	2.26	2.12	0.155	0.02	0.62	0.03	0.26
8:0	1.09	0.83	0.91	1.02	1.04	0.103	0.01	0.38	0.01	0.56
10:0	3.00	2.61	2.72	3.15	3.05	0.242	0.12	0.99	0.03	0.53
12:0	3.29	3.15	3.20	3.54	3.62	0.220	0.53	0.74	0.03	0.97
13:0 <i>iso</i>	0.03	0.05	0.05	0.03	0.06	0.018	0.59	0.36	0.97	0.52
13:0 <i>anteiso</i>	0.08	0.08	0.07	0.08	0.08	0.007	0.75	0.58	0.52	0.18
13:0	0.08	0.11	0.10	0.08	0.13	0.014	0.14	0.16	0.92	0.05
14:0 <i>iso</i>	0.09	0.07	0.08	0.09	0.09	0.016	0.21	0.65	0.39	0.66
14:0	10.6	10.1	10.5	10.9	10.9	0.26	0.09	0.48	0.02	0.43
15:0 <i>iso</i>	0.49	0.45	0.47	0.47	0.41	0.028	0.24	0.37	0.33	0.10
15:0 <i>anteiso</i>	1.04	1.23	1.21	0.92	1.15	0.112	0.16	0.30	0.11	0.23
15:0	0.95	0.97	1.01	1.03	1.09	0.047	0.70	0.31	0.09	0.96
16:0 <i>iso</i>	0.20	0.19	0.22	0.21	0.18	0.024	0.41	0.33	0.48	0.31
16:0	23.2	22.8	23.2	23.7	23.8	1.06	0.68	0.92	0.26	0.71
17:0 <i>iso</i>	0.47	0.45	0.47	0.44	0.44	0.016	0.35	0.46	0.09	0.55
16:1 and 17:0 <i>anteiso</i>	1.67	1.96	1.80	1.52	1.72	0.170	0.13	0.86	0.11	0.26
17:0	0.48	0.47	0.50	0.50	0.47	0.029	0.73	0.91	0.96	0.35
18:0	9.82	9.11	9.63	10.15	9.41	0.449	0.26	0.77	0.42	0.20
18:1 <i>trans</i> -4 and -5	0.06	0.03	0.03	0.03	0.03	0.014	0.13	0.54	0.41	0.60
18:1 <i>trans</i> -6 and -8	0.57	0.67	0.53	0.56	0.37	0.067	0.28	0.03	0.07	0.74
18:1 <i>trans</i> -10	1.52	1.87	1.55	1.65	2.06	0.445	0.50	0.87	0.78	0.39
18:1 <i>trans</i> -11	2.23	2.49	2.22	2.04	1.73	0.207	0.38	0.19	0.04	0.91
18:1 <i>trans</i> -12	0.77	0.78	0.71	0.72	0.69	0.033	0.71	0.15	0.18	0.44
18:1 <i>cis</i> -9	18.7	19.3	19.4	18.0	18.3	0.723	0.45	0.51	0.04	0.70
18:1 <i>cis</i> -11	0.47	0.52	0.47	0.45	0.43	0.023	0.10	0.21	0.01	0.74
18:1 <i>cis</i> -12	0.51	0.64	0.57	0.54	0.61	0.044	0.01	0.96	0.37	0.09
18:1 <i>cis</i> -13	0.81	0.78	0.74	0.71	0.62	0.050	0.71	0.17	0.06	0.68
18:1 <i>cis</i> -15	0.38	0.35	0.38	0.37	0.35	0.015	0.22	0.93	0.59	0.10
18:2 <i>cis</i> -9, <i>cis</i> -12	3.80	4.48	4.25	4.02	4.03	0.172	<0.01	0.56	0.02	0.30
18:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12	0.07	0.12	0.07	0.07	0.10	0.025	0.11	0.52	0.62	0.15
20:1	0.30	0.27	0.30	0.30	0.25	0.023	0.30	0.73	0.56	0.12
18:2 <i>cis</i> -9, <i>trans</i> -11	1.06	1.10	1.11	0.97	0.91	0.092	0.75	0.83	0.06	0.90
18:2 <i>trans</i> -10, <i>cis</i> -12	0.03	0.03	0.03	0.02	0.02	0.007	0.71	0.58	0.31	0.69
22:0	0.08	0.10	0.07	0.09	0.16	0.030	0.74	0.52	0.13	0.07
22:1	0.02	0.03	0.04	0.04	0.01	0.012	0.62	0.47	0.74	0.12
22:6	0.06	0.06	0.06	0.06	0.06	0.006	0.99	0.74	0.74	0.97
24:1	0.05	0.05	0.05	0.05	0.05	0.008	0.57	0.73	0.72	0.94
24:0	0.03	0.02	0.02	0.04	0.02	0.008	0.61	0.48	0.44	0.36
20:5	0.02	0.03	0.02	0.02	0.04	0.011	0.52	0.77	0.74	0.17
20:3n-3	0.005	0.003	0.002	0.004	0.007	0.0042	0.56	0.76	0.41	0.56
20:3n-6	0.18	0.16	0.16	0.19	0.15	0.016	0.39	0.29	0.49	0.44
Other	4.48	4.76	4.81	4.65	4.42	0.173	0.25	0.68	0.15	0.41
ΣOBCFA ³	5.60	6.03	5.99	5.37	5.84	0.272	0.20	0.44	0.16	0.36
Σde novo (<C16)	28.7	27.3	27.4	29.3	29.3	1.07	0.18	0.97	0.01	0.86
Σpreformed (>C16)	43.0	44.5	43.9	42.4	41.9	1.27	0.30	0.75	0.03	0.78
Σ16	25.1	25.0	25.2	25.4	25.7	1.15	0.88	0.90	0.48	0.90

¹HF-C = high-forage and low-starch diet; LF = low-forage and high-starch diet; LF-C = low-forage and high-starch diet with no supplementation; LF-HMTBa = LF-C diet supplemented with HMTBa; LF-IA = LF-C diet supplemented with IA; LF-COMB = LF-C diet supplemented with HMTBa and IA.

²CON = HF-C vs. LF-C; HMTBa = main effect of HMTBa in the LF diets; IA = main effect of IA in the LF diets; INT = interaction of HMTBa with IA.

³Sum of all odd- and branched-chain fatty acids.

Table 4. Effects of supplementing a diet inducing milk fat depression with 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa) and isoacids (IA) on nitrogen utilization and excretion of lactating cows (n = 10)¹

Item	Diet ²					SEM	P-value ³			
	HF-C	LF					CON	HMTBa	IA	INT
		LF-C	LF-HMTBa	LF-IA	LF-COMB					
N intake, g/d	620.5	663.0	650.4	650.2	638.3	29.80	0.01	0.25	0.24	0.97
Milk										
N, g/d	160.6	166.7	177.8	167.2	163.5	35.38	0.44	0.82	0.45	0.39
N, % of N intake	25.7	25.4	27.1	25.5	25.3	4.59	0.83	0.64	0.63	0.50
Feces										
N, g/d	236.8	234.3	233.4	230.3	225.1	15.87	0.84	0.69	0.42	0.78
N, % of N intake	38.1	35.1	35.8	35.5	35.2	1.29	0.06	0.87	0.90	0.61
Urine										
N, g/d	204.4	203.4	201.6	201.9	203.0	11.49	0.90	0.95	0.99	0.77
N, % of N intake	33.2	30.9	31.4	31.5	32.3	2.56	0.05	0.50	0.38	0.84
Urea N, g/d	87.4	82.6	83.6	85.2	86.8	6.72	0.39	0.76	0.50	0.94
N retained, g/d	18.8	58.5	42.1	50.7	46.7	16.69	0.01	0.32	0.87	0.53
N retained, % of N intake	2.89	8.58	6.33	7.38	7.16	3.427	0.05	0.46	0.91	0.54

¹Data of N intake measured during total collection of feces and urine were used (last 3 d in each period).

²HF-C = high-forage and low-starch diet; LF = low-forage and high-starch diet; LF-C = low-forage and high-starch diet with no supplementation; LF-HMTBa = LF-C diet supplemented with HMTBa; LF-IA = LF-C diet supplemented with IA; LF-COMB = LF-C diet supplemented with HMTBa and IA.

³CON = HF-C vs. LF-C; HMTBa = main effect of HMTBa in the LF diets; IA = main effect of IA in the LF diets; INT = interaction of HMTBa with IA.

11% compared with HF-C, which was less severe than biohydrogenation-induced MFD observed in previous studies (20 to 30% decreases in milk fat yield) with similar dietary changes (Baldin et al., 2018; Pitta et al., 2020). As expected, the lower MFD for LF-C likely occurred because our cows were low-producing (average 29 kg/d milk yield) and therefore less susceptible to MFD when an MFD-inducing diet was fed, compared

with high-producing cows (Baldin et al., 2018). Another reason for the moderate MFD for LF-C could be because supplemental PUFA (safflower oil) was included in the HF-C diet at the same inclusion rate as in LF-C. Ramirez Ramirez et al. (2015) reported that diet fermentability (increasing starch from about 19 to 23%) and oil supplementation (soy oil) had similar contributions to severe MFD. In that study, relatively

Table 5. Effects of supplementing a diet inducing milk fat depression with 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa) and isoacids (IA) on nutrient intake and apparent digestibility in lactating cows (n = 10)¹

Item	Diet ²					SEM	P-value ³			
	HF-C	LF					CON	HMTBa	IA	INT
		LF-C	LF-HMTBa	LF-IA	LF-COMB					
Intake, kg/d										
DM	25.0	25.6	26.2	26.0	25.8	1.06	0.25	0.62	0.91	0.38
OM	23.3	24.0	24.6	24.3	24.1	1.07	0.18	0.65	0.80	0.33
CP	3.96	4.12	4.15	4.13	4.10	0.190	0.12	0.99	0.79	0.64
NDF	8.02	7.32	7.52	7.43	7.33	0.338	<0.01	0.65	0.71	0.22
Apparent digestibility, %										
DM	63.0	64.0	64.1	64.3	65.2	1.02	0.44	0.49	0.38	0.64
OM	64.2	65.3	65.3	65.6	66.5	1.00	0.35	0.52	0.33	0.53
CP	63.3	64.6	64.4	64.6	65.8	1.29	0.37	0.60	0.43	0.40
NDF	45.6	42.4	43.3	42.0	44.6	1.67	0.12	0.19	0.73	0.52

¹Data of intake measured during total collection of feces were used (last 3 d in each period).

²HF-C = high-forage and low-starch diet; LF = low-forage and high-starch diet; LF-C = low-forage and high-starch diet with no supplementation; LF-HMTBa = LF-C diet supplemented with HMTBa; LF-IA = LF-C diet supplemented with IA; LF-COMB = LF-C diet supplemented with HMTBa and IA.

³CON = HF-C vs. LF-C; HMTBa = main effect of HMTBa in the LF diets; IA = main effect of IA in the LF diets; INT = interaction of HMTBa with IA.

low-producing cows were used (about 33 and 27 kg/d milk yield in experiments 1 and 2, respectively), and the basal diet contained relatively high PUFA (2.5% in dietary DM) with corn distillers grain at 20% in the ration (DM basis). Therefore, their increased diet fermentability caused moderate MFD (17 and 13% decrease in milk fat yield in experiments 1 and 2, respectively). Supplemental PUFA was also included in HF-C in this study, because our objective was to determine whether alternate biohydrogenation is enhanced when feeding a diet with low NDF and high starch, causing moderate MFD. Because of the same PUFA content between LF-C and HF-C, the possibilities for the moderate MFD observed for LF-C could be either increased diet fermentability (changes in ruminal pH and VFA profile), alternate biohydrogenation (e.g., *trans*-10 C18 isomers) enhanced by increased diet fermentability, or both.

Ruminal biohydrogenation capacity (at least first step of the biohydrogenation) may have been lower for LF-C versus HF-C due to greater concentration of C18:2 *cis*-9,*cis*-12 in milk for LF-C in the rumen. An in vitro study by Troegeler-Meynadier et al. (2014) found decreases in disappearance of C18:2 *cis*-9,*cis*-12 in rumen culture during the short-term incubation (6 h) for high-fermentable substrate and low pH compared with low-fermentable and high pH, respectively. Milk FA concentrations of C18:1 *trans*-10 and C18:2 *trans*-10,*cis*-12 were not different between LF-C and HF-C, suggesting that effects of altered ruminal biohydrogenation of PUFA on MFD were minor. This finding is also supported by the lack of differences in the concentrations of major biohydrogenation intermediates (C18 *trans*-10 and 11 isomers) in the rumen (6 h after feeding) shown in our companion study (Lee et al., 2021). However, the concentrations of various FA that are synthesized de novo in mammary glands were lower for LF-C versus HF-C, probably because of the short supply of precursors for FA synthesis in the mammary gland (e.g., acetate; Urrutia et al., 2019). In our companion study (Lee et al., 2021), LF-C decreased ($P = 0.10$) molar concentration of acetate and increased ($P = 0.01$) propionate in the rumen compared with HF-C, decreasing ($P = 0.03$) the ratio of acetate to propionate from 3.14 to 2.74. This decreased ratio is also supported by a numerical decrease in total-tract NDF digestibility by 3 percentage-units for LF-C versus HF-C. Therefore, we concluded that the enhanced alternate biohydrogenation producing more C18 *trans*-10 isomers did not occur, and, instead, the moderate MFD for LF-C likely resulted from changes in rumen fermentation (pH and VFA profile) by increased diet fermentability. Changes in rumen bacterial community (α -diversity and relative

abundances of 16S rRNA gene sequences at phylum and genus levels) by LF-C versus HF-C are shown in our companion study (Lee et al., 2021). Despite the changes in rumen bacterial diversity and community, the sum of all the odd- and branched-chain FA in milk, which may be a potential marker for rumen microbial changes (Vlaeminck et al., 2006), was not different between LF-C and HF-C. Inconsistent results about changes in milk odd- and branched-chain FA during MFD have previously been observed (Ramirez Ramirez et al., 2016; Baldin et al., 2018; Pitta et al., 2020).

Cows fed the LF-C diet had greater DMI compared with HF-C. Complex factors are involved in regulating DMI (Allen, 2000), and the following 2 factors could be mainly associated with changes in DMI: increasing supply of rumen fermentable energy in a ration can decrease DMI (hepatic oxidation theory), and decreasing forage NDF can reduce gut fill and increase DMI (Allen, 2000). The latter may have been the case in this study. A 4% increase in milk protein yield for LF-C versus HF-C is in agreement with some (Sánchez-Duarte et al., 2019) but not all studies when diet fermentability was increased (Weiss et al., 2009). Body protein accretion in addition to milk protein yield may have increased as well for LF-C versus HF-C, as evidenced by lower N excretion in feces and urine (as percent of N intake), which resulted in increased N retention. However, BW and BW change do not support body protein accretion. Despite the increase in milk protein yield, ECM was decreased for LF-C due to a 12% decrease in milk fat yield compared with HF-C. In consequence, this resulted in decreased feed efficiency (ECM/DMI, kg/kg).

Main Effect of HMTBa Within the LF Diets

Supplementation of the LF diets with HMTBa (main effect of HMTBa) did not alter milk fat concentration but tended to increase milk yield from 28.4 to 30.1 kg/d, which resulted in increasing milk fat yield. We also observed no main effect of HMTBa in milk concentrations of C18:1 *trans*-11 and C18:2 *trans*-10,*cis*-12, which did not agree with our hypothesis. Baldin et al. (2018) observed changes in milk fat concentration of C18:1 *trans*-11 and C18:1 *trans*-10 (increasing by 35% and decreasing by 50%, respectively) when an MFD-inducing diet was supplemented with HMTBa (28.5% NDF, 29.2% starch, and 3.1% PUFA in dietary DM), alleviating MFD. In that study, however, MFD did not occur when the MFD-inducing diet was fed to low-producing cows (about 30 kg/d milk yield), and HMTBa had no effect on milk concentrations of C18 *trans*-10 and *trans*-11 isomers. This explains, at least in

part, the minimal changes in milk FA profile observed in response to HMTBa supplementation of a moderate MFD-inducing diet fed to relatively low-producing cows (30 kg/d milk yield) in the current study and indicates that the major factor causing moderate MFD could be other dietary factors, rather than an increase in biohydrogenation intermediates causing severe MFD. Previously, milk yield increased when cows were fed HMTBa in some studies (Hansen et al., 1991; Piepenbrink et al., 2004) but not others (Zanton et al., 2014; Feng et al., 2018). As a methionine precursor, it has been speculated that HMTBa has postabsorptive effects on milk yield and milk fat synthesis by serving as a methyl donor and increasing blood triglycerides rather than acting solely as a protein building block (Huber et al., 1984; Zanton et al., 2014). These potential postabsorptive effects of HMTBa may have alleviated MFD in the current study, and are supported by the lack of a main effect of HMTBa on total-tract NDF digestibility within the LF diets. However, ruminal effects of HMTBa on alleviating MFD cannot be ruled out, although minimal changes in rumen bacterial diversity and community (amplicon sequencing variants) were observed in response to HMTBa supplementation in our companion study (Lee et al., 2021). We discussed an increase in mass of rumen bacteria in our companion study (Lee et al., 2021) as a potential ruminal effect of HMTBa to alleviate MFD according to increased concentration of saturated FA and decreased concentrations of poly- and monounsaturated FA. An increase in microbial protein synthesis was observed previously when HMTBa was provided to lactating cows (Lee et al., 2015).

The observation of no changes in milk C18 *trans*-10 and 11 isomers for HMTBa under MFD contrasts with the results from Baldin et al. (2018) and Pitta et al. (2020). Again, this discrepancy can be partly explained by productivity of cows (i.e., high-producing cows vs. low-producing cows), as previously discussed. The reason for differing effects of HMTBa supplementation on milk FA profile when fed to low- and high-producing cows is not known. However, DMI for low-producing cows is much lower (about 12%), altering rumen passage rate and feeding and ruminating behaviors, which might cause different responses to an MFD-inducing diet (Jenkins et al., 2008; Baldin et al., 2018). In the current study, because of an increase in milk fat with HMTBa supplementation, ECM production increased and ECM/DMI (kg/kg) tended to increase with HMTBa supplementation of LF diets. Milk protein content and yield were not affected by HMTBa. Supplemental HMTBa is a methionine precursor and often increases milk protein yield (Zanton et al., 2014), but not always

(Lee et al., 2015). Lack of effect of HMTBa on milk protein yield may indicate that methionine was not limiting for milk protein synthesis for the late-lactation cows used in this study. This is supported by the lack of a main effect of HMTBa on N metabolism (i.e., dietary N utilization and excretion) in the LF diets.

Main Effects of IA Within the LF Diets

As the main effect, supplementation of IA increased milk fat yield within the LF diets. This occurred via a mechanism which is different from the mechanism for HMTBa. Supplementation of IA increased milk fat yield by increasing milk fat content but not milk volume, and the increased milk fat content resulted from increased de novo synthesis of milk FA. Increased milk fat yield is consistent with results from most studies in which individual BCVFA, IA, or IA without valerate were fed to cows (Papas et al., 1984; Peirce-Sandner et al., 1985; Wang et al., 2019). Similar results were also observed in a study by Liu et al. (2018). In that study, a diet with 42% NDF and an estimated 24% starch (starch concentration not reported) was supplemented with IA (without valerate; 0, 30, 60, and 90 g/d). Increasing IA supplementation linearly increased milk fat content and yield and de novo synthesis of milk FA. In the current study, we detected no main effect of IA on C18:2 *trans*-10,*cis*-12 or C18:1 *trans*-10 in milk fat, directly inhibiting de novo FA synthesis (Jenkins and Harvatine, 2014). Therefore, the increase in de novo synthesized FA in milk fat for IA could be the main reason for MFD alleviation for IA. However, in our companion study (Lee et al., 2021), we observed increased concentrations of C18:2 *trans*-10,*cis*-12 and C18:1 *trans*-10 and decreased the concentration of C18:2 *cis*-9,*trans*-11 in the rumen (6 h after feeding) for IA supplementation within the LF diets. The increase in C18 *trans*-10 isomers in the rumen did not affect milk C18 *trans*-10 isomers, although C18 *trans*-11 isomers were decreased in milk for IA. This may suggest that ruminal concentration does not reflect the flow to and absorption in the small intestine, or that although IA supplementation increased alternate biohydrogenation in the rumen, the greater production of C18 *trans*-10 isomers in the rumen was not fully transferred to the mammary gland, so that direct inhibition of de novo synthesized FA by C18 *trans*-10 isomers did not occur. We do not know whether IA lowers the transfer of ruminal C18 *trans*-10 isomers into milk. However, IA increased rumen bacterial diversity and the diversity of functional features (i.e., enzymes; Lee et al., 2021), suggesting, at least in part, greater biohydrogenation in the rumen on a daily basis. This is likely supported

by low concentration of linoleic acid (C18:2 *cis*-9,*cis*-12; major PUFA supplemented) in milk fat.

Although IA supplementation increased bacterial diversity, functional features in the rumen (Lee et al., 2021), and milk fat yield within the LF diets, the numerical decrease in NDF digestibility (3.2 percentage-unit decrease) for LF-C versus HF-C was not alleviated by IA supplementation. The addition of IA (especially BCVFA) has been shown in several *in vitro* (Gorosito et al., 1985; Yang, 2002) and *in vivo* (Liu et al., 2018; Wang et al., 2019) studies to increase fiber digestibility because BCVFA can stimulate growth of cellulolytic bacteria in the rumen (Wang et al., 2019). Most studies that observed increased fiber digestibility with supplemental IA used diets with high NDF (e.g., >40% of dietary DM; Liu et al., 2018; Wang et al., 2019). When BCVFA were infused intraruminally to lactating dairy cows fed a diet consisting of <30% NDF of dietary DM, Klusmeyer et al. (1987) detected no difference in NDF digestibility, suggesting that stimulation of fiber degradation by IA could be related to fiber concentration in the diet. Furthermore, the lack of effect of IA on NDF digestibility may have been associated with dietary protein supply. In a study by Wang et al. (2018), when 2-methylbutyrate was provided to steers fed diets at 2 different dietary protein concentrations (about 10 and 13% in dietary DM), an interaction between 2-methylbutyrate and CP levels for NDF digestibility of corn straw (measured by an *in situ* technique in steers fed the treatment diets) was observed. The interaction indicated that NDF digestibility did not respond to 2-methylbutyrate supplementation with the high-CP diet, whereas NDF digestibility increased with the low-CP diet supplemented with 2-methylbutyrate. In the current study, the lack of an IA effect on NDF digestibility could be due to dietary protein supply (especially RDP; 10% of DM) being sufficient according to NRC (2001).

Potential postabsorptive effects of IA on increasing milk fat yield cannot be ruled out. In a recent study by Wang et al. (2019), supplementing 80 g/d of BCVFA (i.e., IA without valerate) increased growth hormone concentration in plasma and increased milk fat content and yield. In another study by Liu et al. (2008), supplementing isobutyrate at an increasing rate of 20, 40, and 60 g/d also linearly increased growth hormone concentrations in plasma. This increase in growth hormone agrees with the conclusion of the review by Andries et al. (1987). Growth hormone can partition nutrients away from body tissues to the mammary gland by decreasing mRNA expression of lipoprotein lipase (Beswick and Kennelly, 2000) and therefore increase the availability of glucose and lipids for milk

synthesis (Hart, 1983; Bauman, 1999). In agreement, supplementing BCVFA at 80 g/d increased expression of mRNA of key enzymes that are related to FA synthesis in mammary glands and increased *de novo* synthesis of FA (Eppard et al., 1985; Liu et al., 2018). Increased *de novo* synthesized FA in milk fat was observed in the current study. However, milk protein yield was not affected by IA supplementation within the LF diets, and neither dietary N excretion nor retained N were affected, suggesting that N metabolism was less responsive to IA in cows fed a moderate MFD-inducing diet or a diet with sufficient dietary protein supply according to NRC (2001). Liu et al. (2018) and Wang et al. (2019) reported that milk protein yield increased when cows were fed a diet with IA without valerate, but this could be a result of increased DMI in these studies. Results from previous studies likely suggest that milk protein yield is less responsive to IA when DMI is not altered.

Effects of the Combination of HMTBa and IA Within the LF Diets

We detected interactions between HMTBa and IA on several measures (milk yield, ECM, milk fat yield, and others). Further investigation indicates that the interactions occurred because the combination of HMTBa and IA did not have additive effects on these measures. For example, milk yield was greater for LF-HMTBa versus LF-C, but the difference was diminished when the combination of HMTBa and IA (i.e., LF-COMB) was used. Milk fat yield was increased for LF-HMTBa and LF-IA compared with LF-C, but milk fat yield was not additive for LF-COMB, resulting in a lack of additive effect on ECM as well. Lack of additive effect of LF-COMB does not support our hypothesis, even though supplementation of HMTBa or IA alleviated MFD apparently with different mechanisms. Lack of additive effects between HMTBa and IA were also observed in the rumen (i.e., rumen fermentation and bacterial community and diversity; Lee et al., 2021). For example, an increase in ruminal butyrate (molar proportion) by HMTBa was diminished (interaction $P = 0.03$) when fed in combination with IA. Interactions also occurred ($P \leq 0.09$) between HMTBa and IA on the relative abundances of some rumen bacterial taxa. In terms of ruminal effects, our companion paper (Lee et al., 2021) discusses aminotransferases that are common between BCAA (from BCVFA) and methionine (from HMTBa; Fernández and Zúñiga, 2006) as a potential factor interrupting their co-metabolism by rumen bacteria when both are sufficiently available. Furthermore, because of potential postabsorptive effects of HMTBa and IA on MFD, as discussed previously, physiological

and biological associations between HMTBa and IA cannot be ruled out. However, we did not find potential evidence in the literature for postabsorptive association between HMTBa and IA, which needs to be further studied. In the current study, the use of HMTBa or IA alone was a better strategy to reduce moderate MFD than feeding a combination of HMTBa and IA.

CONCLUSIONS

Decreasing dietary NDF from 32 to 29% (forage NDF from 20.1 to 17.4%) and increasing dietary starch content from 24 to 30% induced moderate MFD in cows producing 30 kg/d of milk. Supplementing an MFD-inducing diet with HMTBa or IA increased milk fat yield, lowering risk of milk fat depression in field situations. However, supplementation of HMTBa increased milk yield without increasing milk fat content, whereas IA increased milk fat content without altering milk yield. Despite the different mechanism of increasing milk fat, the combination of HMTBa and IA did not have additive effects on milk fat yield. More studies are needed to understand biochemical and physiological interactions between HMTBa and IA.

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