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2-Hydroxy-4-(Methylthio)-Butanoate Supplementation Affects Production, Milk Fatty Acid Profile, and Blood Metabolites of High-Producing Holstein Cows

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Abstract: The objectives of this study were to evaluate the effects of supplementing the diet of high-producing Holstein cows with 2-hydroxy-4-(methylthio)-butanoate (HMTBa) on their milk production and composition, milk fatty acid profile, blood metabolites, and body parameters. The study was conducted in a commercial dairy herd in Paraná State, Southern Brazil. One hundred and fifty-eight multiparous cows were used in a randomized block design during 42 experimental days. Cows were distributed into two treatments: the control treatment cows received 100 g/cow/day of corn meal, while the HMTBa-supplemented cows received 35 g of HMTBa + 65 g/cow/day of corn meal. HMTBa supplementation did not alter milk production but improved milk fat content. Cows receiving HMTBa supplementation showed an increase in the concentration of milk medium-chain fatty acids. Serum levels of blood urea and aspartate aminotransferase were lower in HMTBa-supplemented cows. Cows supplemented with HMTBa increased their body condition score. In summary, HMTB supplementation in high-producing Holstein cows improved productive performance, particularly increased milk fat content, altered milk fatty acid profile, and changed some blood metabolites. Our findings contribute to our understanding of using a methionine analogue as a dietary strategy for optimizing milk quality in high-producing Holstein cows.

Keywords: amino acids; fatty acids; methionine analog; metabolizable protein

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1. Introduction

2-hydroxy-4-(methylthio)-butanoate (HMTBa) is a methionine analogue that was initially developed as a precursor in milk protein synthesis [1]. In the rumen environment, it can be absorbed and converted to methionine, becoming a source of this essential amino acid [2]. It can also facilitate fiber degradation in the rumen and serve as a nitrogen source for microbial protein synthesis, improving rumen fermentation [3]. Some studies reported an increase in the reticular nitrogen content and microbial nitrogen production with increasing doses of HMTBa [4]. Subsequent studies have indicated that HMTBa leads to an increase in the production and concentration of milk fat [5,6]. There are also reports of positive effects of HMTBa on the production of volatile fatty acids in the rumen, with

an increase in propionate concentration, possibly as a result of alterations in the rumen fermentation pattern [7].

The main effect of HMTBa, as described in the literature, is the improvement of milk fat production, especially in cows at high dietary risk (high-starch and low-fiber diets) of milk fat depression (MFD) syndrome. The main hypothesis is that HMTBa may stabilize hydrogenation in the rumen, thus preventing unsaturated fatty acids (trans-10) from causing MFD [8]. This syndrome may occur in diets rich in polyunsaturated fatty acids (PUFAs), high in fermentable carbohydrates, and low in fiber, inhibiting de novo synthesis in the mammary gland [9]. In such conditions, HMTBa supplementation mainly affects milk fat production by decreasing the concentration of trans-10 C18:1 in the milk of high-producing Holstein cows [10]. In addition, HMTBa can have an effect on the concentration and secretion of milk fat biomarkers (C15:0, C17:0, and C17:1), originated from microbial nitrogen flux [11]. Nevertheless, the reasons for varying effects of HMTBa supplementation on milk fatty acids profile between low- and high-producing cows remains unknown [12].

It was found that approximately 39.5% of HMTBa escaped rumen degradation, and this percentage was not affected by the amount or form of the methionine hydroxy analog fed [13]. Furthermore, the HMTBa that escaped ruminal degradation was likely absorbed and metabolized to methionine [14]. The effects of HMTBa and methionine on postpartum metabolism and milk fat synthesis have also been discussed [15,16]. Methionine is involved in the transport of lipids, acting as a methyl donor and increasing blood triglycerides, which are a key substrate for milk fat synthesis and milk production [5,9,15,16].

Although there are increasing number of studies exploring the mechanism of action of the HMTBa in the rumen, intestinal absorption, and its effects on the metabolism and performance in dairy cows, its mode of action is still not fully understood [17]. Thus, our hypothesis is that supplementing the diet with 0.12% (of DM) of HMTBa will sufficiently improve the productive performance and alter the milk fatty acid profile of high-producing Holstein cows fed diets containing moderate levels of NDF and starch. Therefore, the aim of this study was to evaluate the effects of supplementing a diet with 2-hydroxy-4-(methylthio)-butanoic acid on milk production and composition, milk fatty acid profile, blood metabolites, body weight (BW), and body condition score (BCS) in high-producing Holstein cows.

2. Materials and Methods

2.1. Cows and Experimental Design

All procedures used in this study were approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Federal University of Paraná (UFPR), Brazil, and conducted under experimental license from National Council for the Control of Animal Experimentation (CONCEA) under protocol No. 35/2020. The study was carried out at Agropecuária Harm, a commercial farm located in Castro, Paraná State, Southern Brazil (24°48′47.0″ S 49°58′11.2″ W), from October to December 2020. The trial involved 158 multiparous Holstein cows, housed in a single free-stall barn. These cows were included in a randomized block design that evaluated the effect of HMTBa supplementation during a 42-day period. Cows were blocked based on parity (secundiparous and multiparous), average milk production at the end of a 3-d covariable pre-trial period, and days in milk (DIM).

Lactating cows, within blocks, were randomly allocated into two treatments: control and HMTBa. During the covariable pre-trial period, the HMTBa cows (n = 79; 130 ± 78 DIM) had a milk production of 47.70 ± 6.95 kg/d (mean ± SD) and the control cows (n = 79; 138 ± 131 DIM) had a milk production of 47.76 ± 6.65 kg/d. For the control treatment, cows received 100 g/d of corn meal, while the HMTBa cows received 35 g of HMTBa + 65 g of corn meal per cow daily. In the HMTBa treatment, the supplement was mixed with corn meal and evenly distributed top-dressed over the total mixed ration (TMR). In the control treatment, the cows received only corn meal evenly distributed over the TMR.

The total experiment period consisted of a covariate pre-trial period of 3 d and a supplementation period of 42 d. Diet were evaluated using NC guidelines [18], considering cows with a BW of 745 kg, producing 47.7 kg of milk/d (Table 1). The supplementation dose was set at 0.12% of DM (35 g) of MFP® (Alimet, Novus of Brazil Inc., Indaiatuba, SP, Brazil) to provide 11.76 g of metabolizable methionine for high-producing Holstein cows consuming 29.3 kg/d (of DM) of feed. This calculation considered a bioavailability of 84% and a ruminal escape rate of 40% [14]. Diet was supplied as a TMR once a day at 1300 h, with a maximal refusal rate of 10% of what was provided. Cows were milked three times a day at 06:00 a.m., 14:00 p.m., and 22:00 p.m. The temperature–humidity index (THI) was measured at 30 min intervals throughout the entire 42-day experimental period, with a mean value was 65.4 (EasyLog® EL-USB-2-LCD, Lascar Electronics, Erie, PA, USA). The average daily period during which THI exceeded 68 was 7 h, typically occurring from 11:00 a.m. to 6:00 p.m.

Table 1. Diet composition formulated to meet the requirements of high-producing multiparous Holstein cows.

Variables	Diet [1]
Diet composition (% DM)	
Corn silage	32.85
Corn grain, ground	18.46
Soybean meal, 46% CP	10.35
Oat and pea silage	5.82
Soybean hulls	5.15
Wet brewery residue	3.80
Cottonseed, whole	3.09
Wheat bran	3.05
Ryegrass haylage	3.03
Wheat straw	3.02
Soybean meal, expellers	2.46
Corn germ	2.18
Sodium bicarbonate	1.45
Brewery yeast	1.21
Limestone	0.82
Mineral mix	0.51
Kaolin, hydrous aluminum silicate	0.62
Vida Lac All Lands	0.33
Sodium chloride	0.27
Sugar	0.27
Urea	0.24
Magnesium oxide	0.31
Yeast	0.26
Mycotoxin adsorbent	0.20
MFP, HMTBa [1]	0.12
Mineral-vitamin mix	0.07
Sulfur 70%	0.03
Dicalcium phosphate	0.03

[1] Control group received 100 g/d of corn meal and control group received 65 g/d corn meal + 35 g/d of HMTBa (MFP-Alimet, Novus of Brazil Inc., Indaiatuba, SP, Brazil).

2.2. Data and Sample Collection

Corn silage, oat silage, and ryegrass haylage samples were collected every two weeks. Samples of the TMR were collected once a week for each treatment, immediately after feeding the diet. Feed and TMR samples were frozen at −20 °C for later analysis. The chemical composition of the diet provided for the experimental cows is shown in Table 2.

Table 2. Chemical composition of the experimental diet.

Variables	Diet [1]
Chemical composition	
Dry matter, %	47.38
Ether extract, %DM	3.98
Neutral detergent fiber, %DM	30.46
Acid detergent fiber, %DM	17.30
Lignin, %DM	2.40
Forage NDF, %	21.29
Ash, %DM	8.98
Starch, %DM	24.98
Crude protein, %DM	16.89
RDP, %DM	10.15
RUP, %DM	6.74
Metabolizable protein available in milk, g/d	1523.4
Methionine, %MP	2.10
Lysine, %MP	6.24
Lys:Met, %MP	2.97

[1] Control group received 100 g/d of corn meal and control group received 65 g/d corn meal + 35 g/d of HMTBa (MFP-Alimet, Novus of Brazil Inc., Indaiatuba, SP, Brazil).

Individual cow BW was measured on the 1st and 42nd days of the experiment, using a graduated tape specific for dairy cattle which considered thoracic perimeter. The BCS was estimated on the same experimental days by two trained technicians on a scale of 1 (severe under-conditioning) to 5 (severe over-conditioning) with 0.25 increments [19].

Milking data were integrated with a central software (DairyPlan® C21 v.5.3, GEA) that generated real-time information on the milk production of each cow at every milking. Milk sample collection for milk composition analysis was undertaken during the last 3 d of treatment periods (19th, 20th, and 21st day), with 21-day intervals. Nine consecutive milkings were followed, with an 8 h interval for each collection period (pre-trial period, first and second supplementation periods). At each milking, individual milk samples were collected and placed in a 40 mL polyethylene flask containing bronopol, a milk conservative. These milk samples were refrigerated at 3 °C before being sent to the laboratory for analysis. Additional milk samples were collected at the 5th milking at 06:00 a.m. (20-d of the second period) to analyze the milk fatty acid profile. These individual milk samples were deposited in small 200-mL bottles and immediately frozen at −18 °C for later analysis.

Blood samples were collected via venipuncture of the coccygeal vessels using Vacutainer tubes (9 mL) with CAT serum Clot Activator (Greiner; Bio-one Ltd., Stonehouse, UK). Individual blood samples were collected once on the 42nd d of the supplementation period, 6 h after providing the treatments [14]. After collection, blood samples were immediately refrigerated. Subsequently, the samples were centrifuged at 3000× *g* for 10 min to obtain serum, which was stored in 1.5 mL microtubes (eppendorf®) and frozen at −20 °C [20].

The forage and TMR samples were dried in a forced circulation oven at 55 °C and ground to 1 mm in a knife mill (Wiley, Thomas Scientific, Swedesboro, NJ, USA). Total dry matter was determined by drying the samples in an oven at 105 °C for 16 h. The nitrogen content of the feeds was determined using the Kjeldahl method with subsequent estimation of crude protein (N × 6.25). Mineral residue was determined by sample incineration at 550 °C for 5.5 h. The neutral detergent fiber content was determined according to [21], using thermostable α-amylase. The acid detergent fiber content was estimated according to [22]. The ether extract of feed samples was determined using ANKONTF[10] (extractor) instruments and petroleum ether. The analyzes were carried out at the Animal Nutrition Laboratory of the Federal University of Paraná in Curitiba, Brazil.

Concentrations of milk fat, total protein, lactose, total solids, casein, and milk urea nitrogen were determined in the Centralized Milk Laboratory from the Holstein Breeders Association of Paraná State (APCBRH) using mid-infrared spectrometry methodology (Nexgen, Bentley Instruments®; Chaska, MN, USA), whereas somatic cell count (SCC) was

determined in the same laboratory using flow cytometry. Fat, total protein, lactose, total solids, and casein values were also expressed as absolute values (in kg/d), multiplying their average contents by the daily milk production. The fat-corrected milk (4% FCM) yield was calculated using the Gaines formula [23] as follows: FCM = 0.4 × milk yield (kg) + 15 × fat yield (kg). The average daily energy-corrected milk per cow was calculated using the ECM formula [24]: ECM (kg): milk yield (kg) × {[38.30 × fat content (g/kg) + 24.20 × protein content (g/kg) + 16.54 × lactose content (g/kg) + 20.7]/3140}.

Milk fatty acids profile was determined using the extraction and methylation procedure [25], with separation via gas chromatography (Focus CG-Finnigan) using a CP-Sil 88 (Varian) capillary column (100 m × 0.25 mm × 0.2-µm) film thickness. An aliquot of 1 µL of the esterified extract was injected into the chromatograph and the identification of the fatty acids was made by comparing the retention times and the percentages of the fatty acids obtained through the software Chromquest 4.1 (Thermo Electron, Parma, Italy). Fatty acids were identified by comparing the retention times of methyl esters in the samples with fatty acid standards. The standard from Supelco TM Component FAME Mix, cat 18,919 was used. This analysis was carried out in Animal Nutrition and Growth Laboratory (LNCA) of the University of São Paulo (USP), Piracicaba, Brazil.

Blood serum samples were stored at −20 °C until subsequent analysis via the colorimetric enzymatic method using commercial kits for glucose (K082-5.1; Bioclin, Belo Horizonte, Brazil), triglycerides (K117-5.1; Bioclin, Belo Horizonte, Brazil), cholesterol (K083-5.1; Bioclin, Belo Horizonte, Brazil), bilirubin (K106-1.1; Bioclin, Belo Horizonte, Brazil); analysis via the kinetic ultraviolet method for urea (K056-4.1; Bioclin, Belo Horizonte, Brazil), aspartate aminotransferase (AST) (K048-7.3; Bioclin); analysis via the biuret method for total protein (K031-3.1; Bioclin, Belo Horizonte, Minas Gerais); and analysis via the bromocresol green method for albumin (K040-3.1; Bioclin, Belo Horizonte, Minas Gerais). The analyses were performed in an automatic biochemical analyzer (Mindray BS-200®; Bath, UK). Hemolyzed samples were discarded. Biochemical analyzes were carried out at the Veterinary Clinical Pathology Laboratory of the Federal University of Paraná, Curitiba, Brazil.

2.3. Statistical Analysis

Data residuals were assessed for normality and homogeneity of variance via histograms and statistical tests as part of the UNIVARIATE procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Since somatic cell count data did not follow a normal distribution, they was transformed into a linear scale through log transformation: SCS = log2 (SCC/100) + 3 [26]. Milk production and composition, somatic cell count linear score, and milk urea nitrogen data were analyzed using the MIXED procedure of SAS. The model considered the fixed effects of treatment (Control or HMTBa), time (day), and their interactions; it also included the covariate and the random effects of block and cow within treatment. The model with the lowest Akaike information criteria corrected (AICC) value was selected. Results are presented as least squares means, and multiple mean comparisons were performed using Tukey's test. For BW, BCS, milk fatty acid profile, and blood metabolites data, which did not involve repeated measures, the model included the effects of treatment (Control vs. HMTBa) and the random effects of block and cow within treatment. Statistical significance was declared at $p \leq 0.05$, while a trend was declared at $0.05 < p \leq 0.10$.

3. Results

The effects of supplementing a diet with HMTBa on BW, BCS, and changes in BW and BCS (ΔBW and ΔBCS, respectively) in high-producing Holstein cows are shown in Table 3. Cows that received a daily supplementation of 35 g/d of HMTBa for 42 d tended to have greater ΔBW (+17.6 kg vs. +7.6 kg; $p = 0.09$) and an increased ΔBCS (+0.28 vs. −0.04; $p < 0.01$) compared to control cows.

Table 3. Changes in body weight and body condition score of high-producing Holstein cows supplemented with a diet with 2-hydroxy-4-(methylthio)-butanoic acid.

Variables	Treatment			
	Control [1]	HMTBa [2]	SEM	*p*-Value
Body weight				
D1	745	733	6.8	0.21
D42	753	751	6.6	0.89
ΔBW [3]	+7.6	+17.6	5.8	0.09
Body condition score				
D1	3.27	3.08	0.06	<0.01
D42	3.23	3.36	0.08	0.12
ΔBCS [4]	−0.04	+0.28	0.09	<0.01

[1] Control group received 100 g/d of corn meal. [2] Treatment group received 65 g/d corn meal + 35 g/d of HMTBa (MFP-Alimet, Novus of Brazil Inc., Indaiatuba, SP, Brazil). [3] ΔBW = Change in body weight. [4] ΔBCS = Change in body condition score.

The effects of supplementing the diet with HMTBa on milk production and composition in high-producing Holstein cows are shown in Table 4. There were no significant differences between the control and HMTBa treatment for milk production ($p = 0.11$) and 4% FCM ($p = 0.32$), but higher ECM yield was observed for treated cows (49.12 vs. 47.27 kg/d; $p < 0.01$). Cows that received HMTBa supplementation showed higher milk fat content (3.94 vs. 3.75%; $p < 0.01$), milk lactose content (4.74 vs. 4.69%; $p = 0.01$), and milk total solids content (13.03 vs. 12.86%; $p = 0.05$). On the other hand, the HMTBa cows showed lower somatic cell score in the milk (2.1 vs. 2.5; $p = 0.05$). No significant differences were observed between control and HMTBa-supplemented cows for milk protein content ($p = 0.52$), milk protein production ($p = 0.21$), milk casein content ($p = 0.35$), and milk urea nitrogen (MUN) ($p = 0.54$). Interactions between treatment (HMTBa vs. control) and time (sampling days) effects are also shown in Table 4.

Table 4. Milk production and composition in high-producing Holstein cows supplemented with a diet with 2-hydroxy-4-(methylthio)-butanoic acid (HMTBa).

Variables	Treatment			*p* Value [3]		
	Control [1]	HMTBa [2]	SEM	Treat	Time	Treat × Time
Milk Yield kg/d	46.77	49.41	0.90	0.11	0.001	<0.01
4% FCM kg/d	47.42	49.03	0.81	0.32	<0.001	0.01
ECM kg/d	47.27	49.12	0.47	<0.01	<0.001	<0.01
Fat, %	3.75	3.94	0.04	<0.01	<0.001	0.10
Total Protein, %	3.25	3.27	0.02	0.52	<0.001	0.28
Lactose, %	4.69	4.74	0.01	0.01	<0.001	0.12
Casein, %	2.59	2.61	0.01	0.35	<0.001	0.03
Total Solids, %	12.86	13.03	0.05	0.05	0.003	0.07
Fat, kg/d	1.91	1.98	0.03	0.13	<0.001	0.02
Total Protein, kg/d	1.55	1.58	0.02	0.21	<0.001	0.01
Lactose, kg/d	2.28	2.28	0.03	0.28	<0.001	<0.01
Casein, kg/d	1.22	1.24	0.01	0.28	<0.001	<0.01
Total Solids, kg/d	6.21	6.27	0.07	0.55	<0.001	<0.01
MUN, mg/dL [4]	17.17	16.96	0.22	0.54	<0.001	<0.01
Somatic Cell Score [5]	2.5	2.1	0.20	0.05	<0.001	0.02

[1] Control group received 100 g/d of corn meal. [2] Treatment group received 65 g/d corn meal + 35 g/d of HMTBa (MFP-Alimet, Novus do Brazil Inc., Indaiatuba, SP, Brazil). [3] *p*-values refer to the ANOVA results for the main effects of treatment (Treat.), time (days), and the interaction between treatment and time. [4] MUN = Milk urea nitrogen. [5] SCS = log2 (SCC/100) + 3 [26].

The effects of supplementing a diet with HMTBa on the milk fatty acid profile in high-producing Holstein cows are shown in Table 5. Cows that received HMTBa supplementation showed increased concentrations of medium-chain fatty acids such as C11:0 (+0.02%; $p = 0.01$), C13:0 (+0.03%; $p < 0.01$), C14:0 (+0.40%; $p = 0.04$), and C15:0 (+0.20%;

$p < 0.01$). Furthermore, HMTBa-supplemented cows showed higher concentrations of odd- and branched-chain fatty acids (3.88 vs. 3.65%; $p < 0.02$) compared to control cows.

Table 5. Composition of milk fatty acids of high-producing Holstein cows supplemented with a diet containing 2-hydroxy-4-(methylthio)-butanoic acid.

Fatty Acid g/100	Control [1]	HMTBa [2]	SEM	*p*-Value
C4:0	2.29	2.30	0.09	0.95
C6:0	1.87	1.91	0.04	0.31
C8:0	1.17	1.20	0.03	0.37
C10:0	3.29	3.36	0.06	0.42
C10:1	0.23	0.24	0.008	0.23
C11:0	0.09	0.11	0.008	0.01
C12:0	3.94	4.03	0.07	0.39
C12:1	0.03	0.03	0.002	0.10
C13:0	0.22	0.25	0.01	<0.01
C13:0 iso	0.03	0.03	0.001	0.36
C14:0	11.36	11.76	0.19	0.04
C14:0 iso	0.07	0.07	0.002	0.90
C14:1 cis-9	0.93	0.94	0.03	0.74
C15:0	1.18	1.38	0.03	<0.01
C15:0 iso	0.19	0.19	0.004	0.66
C15:0 anteiso	0.40	0.40	0.007	0.89
C16:0	33.44	33.74	0.70	0.67
C16:0 iso	0.20	0.20	0.005	0.97
C17:0 iso	0.36	0.36	0.007	0.83
C16:1 cis 9	1.40	1.44	0.05	0.62
C17:0	0.65	0.67	0.01	0.14
C17:1	0.16	0.17	0.001	0.27
C18:0	9.69	9.24	0.32	0.16
C18:1 trans	2.11	2.26	0.09	0.10
C18:1 cis-9	18.18	17.48	0.50	0.17
C18:1 cis-11	0.65	0.63	0.02	0.64
C18:1 cis-12	0.42	0.44	0.01	0.22
C18:1 cis-13	0.05	0.05	0.003	0.87
C18:1 trans-16	0.29	0.29	0.007	0.38
C18:1 cis-15	0.12	0.12	0.004	0.55
C18:2 cis-9, cis-12	2.69	2.70	0.07	0.80
C18:2 cis-9, trans-11	0.39	0.41	0.01	0.24
C20:0	0.11	0.10	0.003	0.05
C18:3-n6	0.15	0.14	0.001	0.14
C18:3-n3	0.07	0.07	0.003	0.45
∑ De Novo (<C16) [3]	25.15	25.78	0.42	0.14
∑ Mixed (C16)	34.87	35.19	0.70	0.65
∑ Preformed (>C16) [3]	35.45	34.85	0.61	0.32
∑OBCFA [4]	3.65	3.88	0.07	0.02
SFA [4]	71.19	71.60	0.76	0.59
MUFA [4]	25.49	24.94	0.58	0.35
PUFA [4]	3.49	3.50	0.09	0.91
UFA [4]	28.69	28.31	0.75	0.36

[1] Control group received 100 g/d of corn meal. [2] Treatment group received 65 g/d corn meal + 35 g/d of HMTBa (MFP-Alimet, Novus do Brazil Inc., Indaiatuba, SP, Brazil). [3] FA < 16 C from de novo synthesis in the mammary gland, FA > 16 C from dietary or extracted from plasma, and 16 C FA originate from both sources (mixed). [4] Sum of all odd- and branched-chain fatty acids; saturated fatty acids; monounsaturated fatty acids; polyunsaturated fatty acids; and unsaturated fatty acids.

The effects of supplementing a diet with HMTBa on blood metabolites in high-producing Holstein cows are shown in Table 6. Blood urea concentration was lower in the HMTBa-supplemented cows compared control cows (41.3 vs. 46.6 mg/dL; $p < 0.01$). Additionally, HMTBa supplementation resulted in a decrease in the aspartate amino transferase activity (89.4 vs. 105.6 U/L; $p < 0.01$). There were no significant differences between

the HMTBa-supplemented cows and the control cows for serum glucose concentrations (p = 0.49), triglycerides (p = 0.62), cholesterol (p = 0.51), total protein (p = 0.50), albumin (p = 0.59), and bilirubin (p = 0.17).

Table 6. Blood metabolites of high-producing Holstein cows supplemented with a diet containing 2-hydroxy-4-(methylthio)-butanoic acid.

Variables	Treatment			
	Control [1]	HMTBa [2]	SEM	*p*-Value
Glucose, mg/dL	64.3	65.0	0.78	0.49
BUN [3], mg/dL	46.6	41.3	1.06	<0.01
AST, U/L	105.6	89.4	5.44	<0.01
Triglycerides, mg/dL	6.4	6.3	0.20	0.62
Cholesterol, mg/dL	224.8	228.2	6.75	0.51
Total protein, g/dL	7.6	7.5	0.13	0.50
Albumin, g/dL	3.3	3.4	0.04	0.59
Bilirubin, mg/dL	0.07	0.08	0.01	0.17

[1] Control group received 100 g/d of corn meal. [2] Treatment group received 65 g/d corn meal + 35 g/d of HMTBa (MFP-Alimet, Novus do Brazil Inc., Indaiatuba, SP, Brazil). [3] BUN = Blood urea nitrogen.

4. Discussion

We aimed to evaluate whether HMTBa supplementation at 0.12% of DM intake would influence the productive performance of high-producing Holstein cows eating a diet containing moderate fiber (30% NDF) and starch (25% DM) inclusions over a 42-day supplementation period. The methionine analogue was supplied in combination with 65 g of corn meal to mask any potential negative impact on feed intake due to odor and flavor. Previous reports have indicated that supplementing with 90 g of HMTBa (liquid) can lead to some degree of feed refusal in dairy cows, even when mixed with 500 g of ground corn [27]. According to a recent metanalysis, an inclusion of 23.1 g/d (0.15% DM) for cows consuming an average of 19.4 kg/d DMI, did not cause any adverse effect [6]. Recently, a combination of 25 g of HMTBa and 225 g of corn meal, top-dressed, was provided without impacting diet acceptance by the cows [7].

In our study, we supplied 35 g/d (equivalent to 11.76 g of metabolizable methionine) of the commercial MFP (Alimet, Novus of Brazil Inc., Indaiatuba, SP, Brazil), which contains HMTBa (0.12% of the DM). This supplementation was provided to cows with an estimated DM intake of 29.3 kg/d. The main effects of HMTBa supplementation are observed in diets with a high risk of causing MFD, characterized by a drastic reduction in the dietary fiber content. Recent studies have achieved these conditions with an average reduction from 32.3 to 28.7% NDF and an average increase in starch from 24.4 to 29.1% [9–12]. In contrast, other studies found no positive effect of HMTBa on milk fat when diets had NDF content greater than 30% and ether extract content lower than 3% [28,29].

Positive effects of HMTBa on BCS changes were found, with cows supplemented with the methionine analogue showing greater gain in BCS over 42-d period. Similar results were found by [10], when cows supplemented with HMTBa showed greater BW gain (+17.8 kg) than those (+8.4 kg) not supplemented within 21 d on a diet not inducing MFD. On the other hand, HMTBa supplementation had no effects on DM, CP, and NEL intakes, final BW, and BW change [30]. While we were unable to measure individual DMI in our study, it is worth noting that previous studies have shown that supplementing with amounts ranging from 25 to 35 g/d of HMTBa generally does not impact DMI when compared to diets without methionine inclusion [4,5]. In addition, HMTBa supplementation had no effect on apparent nutrient digestibility and duodenal digesta and N flows in cows fed diets with low and high metabolizable protein content [7].

Methionine analogue supplementation did not affect milk production or protein and casein contents, but it increased concentrations of milk fat, lactose, total solids, and the ECM yield. These effects may be partially explained by the role of this methionine

analogue in ruminal metabolism. It is important to note that previous studies have also not found positive effects of HMTBa supplementation on milk production and protein content [5,6,29,31–33]. In addition, it is estimated that only 15% of methionine incorporated in milk directly results from the conversion of HMTBa [34].

The positive effect of this methionine analogue on milk fat is a common finding in the literature [5]. A recent study found that HMTBa can alleviate the negative effects of diets low in fiber and high in starch and polyunsaturated fatty acids, especially in high- producing cows under some degree of MFD [9]. Although the mechanism remains unclear, it is believed that HMTBa stabilizes biohydrogenation in the rumen, reducing the production of intermediates that inhibit de novo FA synthesis in the mammary gland, such as CLA trans-10, cis-12 fatty acid [10]. This increase in milk fat may be due to the modulation of biohydrogenation in the rumen and increased production of precursors for milk fat synthesis.

The observed effect on FA from de novo synthesis, particularly an increase in C14:0, suggests that the HMTB impacts the milk fatty acid profile through its effects on mammary gland fat synthesis and the uptake of preformed fatty acids from blood plasma. A recent study indicated that HMTBa supplementation can increase microbial mass without altering the diversity of rumen bacteria population, leading to changes in rumen fermentation and increased production of propionate and butyrate [29]. This improved rumen fermentation could explain the increases in milk fat, lactose, and total solids.

This methionine analogue also led to increased concentrations of odd- and branched-chain fatty acids (OBCFA), mainly C11:0, C13:0, and C15:0. Another study found that supplementation with 25 g of HMTBa in a low-risk MFD diet resulted in higher concentrations of C15:0 (+9.1%) and C17:0 (+20.0%) [9]. When supplemented under conditions of moderate MFD risk, an increase in C11:0, C13:0, C15:0, and C17:0 concentrations may occur [10]. Odd- and branched-chain fatty acid contents are important indexes of rumen fermentation characteristics and microbial matter leaving the rumen [35,36]. In addition, OBCFA were closely correlated with volatile fatty acids and bacterial populations, with cellulolytic bacteria showing stronger correlations with the OBCFA profiles compared to starch-degrading bacteria [37]. In the literature, there are reports indicating that dairy cows supplemented with hydroxy methionine analogue showed an increase in microbial protein synthesis, improved carbohydrate digestibility, alterations in the fermentation products profile, and an increase in the protozoa number [16,38].

We can infer that HMTBa improved the nitrogen and energy balance in the rumen, reducing blood urea nitrogen (BUN) concentrations, since the urea nitrogen content remained high throughout the experimental period. On the other hand, no differences on MUN were found between treatment groups. In dairy cows, BUN, urine, and milk are primarily derived from excess ammonia absorbed through the rumen wall from dietary protein degradation in the rumen and from deamination of amino acids, which may come from metabolizable protein and the body tissue catabolism [34]. Additionally, the combined supplementation of lysine and methionine has been shown to cause a reduction in the concentration of urea N in blood, urine, and milk [39].

The averages for aspartate aminotransferase suggest an effect between treatments; however, these values (105.6 vs. 89.4 U/L) remained within the range of 78 to 132 U/L, which is an indicative of a normal condition [40]. It is possible that the effect of methionine supplementation on AST is more pronounced in cows during the transition period. This period poses a greater metabolic challenge compared to mid-lactation cows. AST is an enzyme that shows a positive correlation with increased milk production by the mammary gland and can serve as an indicator of liver and heart problems. It can also reflect increased gluconeogenic activity and enzymatic activity in liver and muscle tissue [41]. Additionally, elevated AST levels can be associated with liver function alterations due to fatty liver infiltration in dairy cows [42]. But as already mentioned, the experimental cows in this study were not in negative energy balance; in fact, they gained BW and BCS during the trial.

Further research is needed in order to gain a better understanding of the effects of HMTBa in the rumen, including its potential influence on the microbial community structure [7]. Further studies are needed to explore the interactions between HMTBa and the rumen microbiota [11].

5. Conclusions

Cows supplemented with HMTBa over a 42-day period showed higher milk components and increased concentrations of odd- and branched-chain milk fatty acids. These changes were accompanied by changes in body condition score and reduced blood urea nitrogen concentrations. Therefore, it seems that the HMTBa supplementation at 0.12% of DM inclusion can have an impact on the de novo FA synthesis in the mammary gland, as well as the plasmatic FA uptake. Under similar conditions in this study, it appears that HMTBa supplementation may impact the rumen metabolism of high-producing Holstein cows fed a diet with moderate fiber inclusion.

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