Meta-analysis of 2-hydroxy-4-methylthio-butanoic acid supplementation on ruminal fermentation, milk production, and nutrient digestibility

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ABSTRACT

Methionine is considered one of the most important essential AA for milk protein synthesis in dairy cows. Supplementation of unprotected, free Met is nearly 100% degraded by ruminal microorganisms, which complicates supplementation. 2-Hydroxy-4-methylthio-butanoic acid (HMTBa) can be converted to Met in the body and is used as a Met source in dairy production. However, results of published studies assessing the effects of supplementing Met sources, including HMTBa, on performance variables are inconsistent. A meta-analysis was performed to quantitatively summarize the accumulated results of HMTBa supplementation on animal performance and nutrient digestibility. Data pertaining to HMTBa dose, dietary composition, and major performance variables (rumen volatile fatty acid composition, milk production, nutrient digestibility) were collected from 39 articles containing 169 treatment means. Publications were from scientific journals published from 1970 to 2018; 1 internal report from Novus International Inc. (St. Charles, MO) was also included. The HMTBa effects on response variables were analyzed using linear mixed models with random study effects. Other explanatory variables tested included neutral detergent fiber and crude protein percent as well as days in milk. Results showed that HMTBa supplementation increased blood Met concentration and milk fat yield but had no effect on nutrient digestibility. **Key words:** 2-hydroxy-4-methylthio-butanoic acid (HMTBa), meta-analysis, digestibility

INTRODUCTION

Methionine is often a limiting AA for milk protein synthesis (Vyas and Erdman, 2009; Patton, 2010). In addition to its role as a substrate for protein synthesis,

Met is a strong regulator of protein synthesis (Appuhamy et al., 2011) and is involved in transsulfuration and methylation reactions (Brosnan and Brosnan, 2006). Balancing rations with supplemental Met to improve the AA profile could allow for reduced dietary CP, maximizing lactation performance, and minimizing N excretion to the environment.

Free Met in an unprotected form is almost completely degraded by rumen microbes, resulting in minimal or no additional postruminal Met supply. An effective way to increase postruminal Met supply is to feed a Met product that escapes microbial degradation in the rumen. Protection is commonly achieved by chemical modifications or by physically protecting the Met. One example of chemical modification is 2-hydroxy-4-methylthio-butanoic acid (HMTBa), which is a Met analog where the amino group is replaced with a hydroxyl group. The results on whether this modification could effectively protect HMTBa from ruminal degradation are inconsistent. Koenig et al. (1999) reported about 50% of the HMTBa escaped ruminal degradation and became available for postruminal absorption, whereas Jones et al. (1988) observed 99% of the HMTBa was altered or degraded by rumen microbes.

Researchers have conducted numerous studies over the years to investigate the effects of HMTBa supplementation on performance of dairy cows, but results have been highly variable. Some studies have reported HMTBa supplementation increased milk fat yield (Polan et al., 1970; Lundquist et al., 1985) and enhanced rumen microbial activity (Holter et al., 1972; Lee et al., 2015). Some studies have also reported no beneficial effects of supplemental HMTBa (Olson and Grubbaugh, 1974; Wallenius and Whitchurch, 1974). Because of this disparity in the literature, a need and an opportunity exist to test the effects of supplementing HMTBa across all published studies. The meta-analysis done by Zanton et al. (2014b) included 17 papers on HMTBa supplementation, but only lactation responses to supplemental Met sources were analyzed. Their meta-analysis concluded HMTBa supplementation increases

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milk fat yield by 45 g/d and milk protein yield by 13 g/d, suggesting beneficial effects of supplemental HMTBa. Combining previous work and the previous meta-analysis, these data suggest a potential for HMTBa to affect rumen fermentation. More specifically, with increased ruminal microbial activity from supplemental HMTBa, it is possible HMTBa would increase nutrient digestibility and microbial N flow. Thus, a more comprehensive meta-analysis is needed to investigate the effects of supplemental HMTBa on rumen fermentation, milk production, and nutrient digestion.

The objective of this work was to use a meta-regression approach to study the effects of HMTBa supplementation. We hypothesized that supplementation of HMTBa would affect ruminal fermentation, milk production, and nutrient digestibility.

MATERIALS AND METHODS

Data Collection and Preparation

To accomplish the objective, data were collected from different published articles in the literature (see Appendix Table A1 for list of publications). PubMed, Science Direct, AGRICOLA, and Web of Science databases were searched using the keywords combination from group 1 (methionine, methionine hydroxyl analog or MHA, 2-hydroxy-4-methylthio-butanoic acid or HMTBa or HMB, and ALIMET feed supplement) and group 2 (ruminants, dairy cows, cows, or cattle). Literature cited in these papers was also used to look for papers investigating HMTBa effects. A total of 39 articles reporting results from a total of 169 treatments were collected. Among the 39 articles, 3 reported work conducted with beef cattle breeds and the remainder reported findings from dairy cows. Articles that did not report the inclusion rate of HMTBa were not used. Data on dietary nutrient composition, HMTBa dose, DIM, milk yield, milk composition, ruminal VFA concentration, and blood Met concentration were collected from each article. Milk fat and protein yield were calculated using milk yield and fat or protein content when component yields were not reported. Ruminal VFA were converted to a percentage of total VFA, where total VFA was the sum of acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate if reported. The supplemental amount of commercial HMTBa was converted to a pure HMTBa basis using multipliers of 0.88 and 0.84 for liquid and Ca salt forms of HMTBa, respectively. As a categorical variable, DIM at the beginning of the study was grouped into 4 categories (0 = dry period; 1 = first third of lactation, where DIM is less than 100 d after calving; 2 = second third of

lactation, where DIM is between 100 and 200 d after calving; 3 = last third of lactation, where DIM is more than 200 d after calving).

Model Derivation Procedure

All statistical analysis were conducted using R v. 3.1.3 (R Core Team, 2015). The lmerTest package (Kuznetsova et al., 2016) was used to derive linear mixed models with random intercept effects for each study. Independent variables used in the model were HMTBa dose (g/d), CP percent, NDF percent, and DIM category. Initial models generally included all independent variables described above as potential effectors of the response variable. Interactions of HMTBa and each independent variable, as well as quadratic effects of HMTBa, were tested but not significant, and thus were not included in the initial models. Models were derived following the procedure outlined in Roman-Garcia et al. (2016) and White et al. (2016) with minor modification. Briefly, final models were derived using backward elimination of nonsignificant effects, with the parameter possessing the largest *P*-value removed from the model in the subsequent round. The process was repeated until all *P*-values were less than 0.1. Any remaining parameters with variance inflation factors (**VIF**) greater than 10 were removed. The VIF is a measure of the severity of multicollinearity reflecting the degree to which parameter estimate variance is inflated due to collinearity and has been used in previous work as a model selection tool (White et al., 2017). A VIF >10 is often used to indicate unacceptably high multicollinearity (Roman-Garcia et al., 2016) and is the threshold that we used in the current study.

Evaluating Model Performance

Overall variance of the predictions was determined by calculation of concordance correlation coefficients (**CCC**; St-Pierre, 2004)) and mean square error (**MSE**) as described by Bibby and Toutenburg (1977):

$$\text{MSE} = \frac{1}{n} \sum_{i=1}^n (O_i - P_i)^2,$$

where *n* represents the total number of observations, *O_i* the observed value, and *P_i* the predicted value. The MSE was decomposed into error due to random variation, deviation of the regression slope from unity (slope bias), and central bias (mean bias). Root mean square errors (**RMSE**) were obtained by taking the square root of the MSE. This RMSE was expressed as a proportion

Table 1. Summary statistics for the data included in the data set

Item	N ¹	Mean	Median	Minimum	Maximum
Studies	39				
HMTBa dose, ² g/d	169	23.1	9.81	0.00	77.4
DMI, kg/d	148	19.4	18.9	7.50	29.8
BW, kg	101	598	600	343	778
DIM	152	40.3	4.00	−21	300
Feed composition, %					
CP	138	15.7	15.8	7.60	20.4
NDF	72	36.8	36.1	19.0	62.1
Milk production					
Milk, kg/d	154	28.6	27.6	5.76	45.3
Fat, %	152	3.53	3.57	1.69	4.57
Fat yield, kg/d	125	0.98	0.95	0.10	1.80
Protein, %	113	3.21	3.21	2.64	3.81
Protein yield, kg/d	90	0.93	0.91	0.18	1.41
Digestibility, %					
DM	17	66.4	66.6	58.8	77.7
OM	14	63.6	65.0	49.3	77.4
NDF	16	46.8	48.8	15.2	69.9
CP	21	66.7	66.1	58.0	80.1
Rumen VFA, %					
Acetate	42	62.0	61.0	49.6	79.1
Propionate	42	23.7	24.0	12.5	30.3
Butyrate	42	12.6	12.3	5.20	19.2
Valerate	34	1.48	1.45	0.54	2.55
Blood Met, μ M	36	27.2	22.1	14.8	50.0

¹N = number of treatments.²Mean of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) is not including controls.

of the observed mean to estimate the overall prediction error. Inclusion of the random intercept effects for each study improves the accuracy of the final model and likely results in an error range that reflects biological variation or true response variability. However, when applied on a farm, often a mean bias is associated with model prediction and model results unadjusted for study intercepts can be a more realistic reflection of mean biases that might be expected when applying a model on a farm. This is also supported by the idea that prediction errors in mixed model estimations will be underestimated if uncertainty surrounding the future study effects are not accounted for (Renaudeau et al., 2011). In an effort to show the true response variability and a more conservative estimate of how the model might perform on an individual farm or in a naïve prediction situation, RMSE and CCC were reported with (adjusted) and without study effects (unadjusted). The ratio of between-study variance to total variance was also reported.

RESULTS AND DISCUSSION

Descriptive Statistics of the Data

A summary of the descriptive statistics of the data can be found in Table 1. Every variable has a different number of observations according to what was reported

in each study. The minima and maxima document the ranges of each variable within the data set. The HMTBa dose ranged from 0 to 77.4 g/d with a mean of 23.1 g/d. The average of supplemental HMTBa as a percent of DMI was 0.15, where the average of DMI was 19.4 kg/d. The CP and NDF percent ranged from 7.6 to 20.4% and 19 to 62.1% of dietary DM, respectively. Blood Met concentrations ranged from 14.8 to 50 μ M with a mean of 27.2 μ M.

Effects of HMTBa Supplementation on Ruminal Fermentation and Nutrient Digestibility

The final models of ruminal VFA composition (%) and nutrient digestibility (%) with HMTBa dose are presented in Table 2. The effects of supplemental HMTBa on ruminal VFA molar proportions were not significant. As would be expected based on a large body of prior literature (Lundquist et al., 1983; Wang et al., 2010), the molar proportion of acetate was significantly affected by the NDF percent in the diet. Increasing CP percent increased molar proportion of valerate according to the current analysis. Supplementation of HMTBa did not affect DM, OM, NDF, or CP digestibility. The models of DM, OM, and NDF are not presented in the table because of no significant predictors ($P < 0.1$) left in the model after the backward elimination.

Table 2. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) supplementation on ruminal VFA production (% basis) and nutrient digestibility (%)

Variable	Predictor ¹	Estimate	SE	P-value
Acetate, %	Intercept	43.9	11.5	0.01
	NDFpct	0.76	0.36	0.09
Propionate, %	Intercept	20.7	1.83	<0.001
	DIM1	5.61	2.23	0.04
	DIM2	1.83	2.29	0.45
	DIM3	0.64	4.49	0.86
Valerate, %	Intercept	0.09	0.59	0.88
	CPpct	0.09	0.04	0.02
CP, %	Intercept	90.5	9.02	0.0001
	CPpct	-1.45	0.57	0.047

¹DIM1 = first third of lactation, where DIM is less than 100 d after calving; DIM2 = second third of lactation, where DIM is between 100 and 200 d after calving; DIM3 = last third of lactation, where DIM is more than 200 d after calving; CPpct = % of CP of the diet; NDFpct = % of NDF of the diet.

Koenig et al. (2002) observed that, on average, 39.5% of dosed HMTBa escaped the rumen, with 22% appearing in the duodenum and 17.6% apparently absorbed from the omasum. The remaining 60.5% was largely degraded or used by ruminal microorganisms and, to a lesser extent, absorbed across the ruminal epithelium (McCollum et al., 2000). The HMTBa can be used by rumen microbes to synthesize Met or catabolized to provide sulfur and energy (Belasco, 1980). Noftsker et

al. (2003) reported that supplemental Met sources could spare Met precursors, resulting in more efficient protein synthesis or by shifting bacterial species. It has been previously reported that HMTBa supplementation increased microbial protein synthesis, improved carbohydrate digestibility, changed the profile of fermentation products, and increased protozoa numbers (Patton et al., 1970; Gil et al., 1973; Ray et al., 1983); however, we did not observe effects on ruminal fermentation. Due to the low number of observations of microbial N flow, the effects of HMTBa could not be analyzed.

Effects of HMTBa Supplementation on Production Performance

Supplementation of HMTBa had no effect on milk yield, fat percent, protein percent, or protein yield, but fat yield increased (Table 3). Zanton et al. (2014b) reported HMTBa supplementation increased milk fat by 5.38 g/g of MP Met from HMTBa, whereas in the current study HMTBa supplementation increased fat by 5 g/g of MP Met, assuming 60% of Met from HMTBa degraded in the rumen. The beneficial effects of HMTBa supplementation on milk fat have been well characterized in some studies (Polan et al., 1970; Holter et al., 1972; Wang et al., 2010). Patton et al. (1970) reported HMTBa can stimulate rumen polar

Table 3. Effect of 2-hydroxy-4-methylthio-butanoic acid (HMTBa) supplementation on milk production and blood Met concentration

Variable	Predictor ¹	Estimate	SE	P-value
Milk, kg/d	Intercept	28.1	2.84	<0.001
	DIM1	4.62	3.61	0.21
	DIM2	-0.62	3.76	0.87
	DIM3	-7.89	4.21	0.07
Fat, %	Intercept	3.34	0.35	<0.001
	NDFpct	0.01	0.007	0.08
	DIM1	-0.12	0.29	0.68
	DIM2	-0.27	0.28	0.37
Fat, kg/d	DIM3	0.39	0.32	0.25
	Intercept	0.51	0.17	0.002
	HMTBa dose	0.002	0.0007	0.013
	CPpct	0.03	0.01	0.0005
Protein, %	Intercept	3.42	0.11	<0.001
	NDFpct	-0.007	0.003	0.017
Protein, kg/d	Intercept	0.38	0.59	0.52
	CPpct	0.07	0.03	0.05
	NDFpct	-0.007	0.003	0.02
	DIM1	-0.23	0.18	0.23
	DIM2	-0.27	0.19	0.18
	DIM3	-0.67	0.20	0.005
Blood Met, μ M	Intercept	58.4	14.3	0.001
	HMTBa dose	0.41	0.11	0.002
	CPpct	-2.36	0.93	0.03

¹DIM1 = first third of lactation where DIM is less than 100 d after calving; DIM2 = second third of lactation where DIM is between 100 and 200 d after calving; DIM3 = last third of lactation where DIM is more than 200 d after calving; CPpct = % of CP of the diet; NDFpct = % of NDF of the diet.

lipid formation in vitro and suggested this lipid class increases with protozoa growth. The increased formation of specific biohydrogenation intermediates (e.g., *trans*-10 C18:1) exerts antilipogenic effects in the mammary gland of ruminants and is considered to attribute the causal mechanism underlying diet-reduced milk fat depression (Shingfield and Griinari, 2007). Baldin et al. (2018) observed that HMTBa decreased the isomers of the alternate pathway of ruminal biohydrogenation (*trans*-10 C18:1) and increased isomers of the normal pathway of ruminal biohydrogenation (*trans*-11 C18:1). The supplemental HMTBa may contribute to the increased milk fat yield based on these findings.

One gram of HMTBa increased blood Met concentration by 0.41 μ M. Supplemental HMTBa that escapes ruminal degradation is absorbed mostly in the small intestine, is rapidly converted to L-Met, and shows identical metabolism to any other source of L-Met, including intact protein (Huber et al., 1984). This is consistent with previous findings, as Zanton et al. (2014a) reported pulse-dosed intraruminal administration of HMTBa increased serum Met concentrations. Wang et al. (2010) observed that, when diet was supplemented with HMTBa (0.15% DM basis), plasma concentration was increased 45% compared with control (29.4 vs. 48 μ M). With a DMI of 20.7 kg, it was calculated that each gram of HMTBa increased plasma Met by 0.6 μ M. Given the observed blood Met concentration response to HMTBa dose, it could be used as a potential indicator of HMTBa bioavailability in dairy cows. A residual plot of blood Met showed no systematic patterns, indicating relatively good fit of the model.

Model Evaluation

Table 4 lists the fit statistics for the fat yield and blood Met concentration models in which HMTBa were significant. Due to missing values for independent variables, the number of observations used to fit the model ranged from 24 for the blood Met concentration model to 102 for the fat yield model. The observed and predicted means are the same in all cases as would be expected when linear models were fit to the data. Residuals without study effects can better represent error bounds that could be expected in a practical application scenario, but adjusted values can provide better estimates of the true response; residuals are seen in Figure 1 for predicted fat yield and Figure 2 for predicted blood Met. For purposes of discussion, we refer to the adjusted values for linear mixed models. For RMSE, the unadjusted values were all much higher than the adjusted values, as would be expected when study is a significant factor in the model. For CCC, the adjusted values were much higher than unadjusted values. The MSE represents the total variation associated with the predictions, but the value has units that are a square of the predicted values so it is not intuitive to interpret. Simply taking the square root of it yields units that are identical to the predicted value (i.e., RMSE). Comparing the magnitude of RMSE to the observed mean (RMSE as % of mean) values displays the relative accuracy of the model. When RMSE is expressed as a percentage of the observed mean, it is equivalent to a coefficient of variation, and 10% is generally a good representation for a biological measurement (Bibby

Table 4. Evaluation of models predicting 2-hydroxy-4-methylthio-butanoic acid (HMTBa) responses

Item ¹	Fat, kg/d	Blood Met, μ M
N	102	24
Observed mean	1.01	28.5
Predicted mean	1.01	28.5
RMSE, units	0.08	5.82
Unadjusted RMSE, units	0.28	9.11
RMSE, % mean	7.92	20.4
Unadjusted RMSE, %	25.9	32.0
Mean bias, % MSE	0.00	0.00
Slope bias, % MSE	0.88	2.86
Residual error, % MSE	99.1	97.1
CCC	0.96	0.86
Unadjusted CCC	0.65	0.60
$\bar{r}^2$	0.74	0.46

¹N: indicates number of treatments; RMSE: root mean squared prediction error; CCC: concordance correlation coefficient; MSE: mean squared error; $\bar{r}^2$: ratio of between study variance to total variance; RMSE and CCC unadjusted for study effects are also reported.

Figure 1. Residual errors for predictions of fat yield versus predicted yield. Fat yield were predicted using the parameters listed in Table 3, and residuals were calculated as the difference between observe and predicted values.

Figure 2. Residual errors for predictions of blood Met versus predicted concentrations. Blood Met concentrations were predicted using the parameters listed in Table 3, and residuals were calculated as the difference between observed and predicted values.

and Toutenburg, 1977). In our study, the blood Met concentration model had a RMSE of 20.4%, indicating poor fit, which could be due to the low number of available observations. The CCC measures the agreement between observed and predicted values and is somewhat analogous to coefficient of determination. In accordance with the RMSE, the CCC value for blood Met concentration (0.86) was also lower compared with the fat yield models (0.96). By decomposing MSE, we observed that the mean and slope bias were only a small portion, where the residual error is almost 99% of MSE for fat yield model and 97% for blood met model, indicating most of the remaining variance is not due to or explained by the model.

CONCLUSIONS

This meta-analysis evaluated quantitative relationships between HMTBa supplementation and major performance and digestibility variables. Supplementation of HMTBa increased blood Met concentration and milk fat yield. Although HMTBa supplementation increased milk fat yield, further studies are needed to evaluate the mode of action by which this is occurring.

Future independent data can be used to independently evaluate the models derived herein.

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APPENDIX 1

Table A1. List of the publications used in the analyses

Citation	Journal, volume, page range
Polan et al. (1970)	<i>J. Dairy Sci.</i> 53:607–610
Holter et al. (1972)	<i>J. Dairy Sci.</i> 55:460–465
Hutjens and Schultz (1971)	<i>J. Dairy Sci.</i> 54:1637–1644
Rosser et al. (1971)	<i>J. Dairy Sci.</i> 54:1807–1816
Whiting et al. (1971)	<i>J. Dairy Sci.</i> 55:983–988
Olson and Grubaugh (1974)	<i>J. Dairy Sci.</i> 57:695–697
Wallenius and Whitchurch (1974)	<i>J. Dairy Sci.</i> 58:1314–1319
Chandler et al. (1976)	<i>J. Dairy Sci.</i> 59:1897–1909
Bhargava et al. (1977)	<i>J. Dairy Sci.</i> 60:1594–1604
Stokes and Clark (1981)	<i>J. Dairy Sci.</i> 64:1686–1694
Clark and Rakes (1982)	<i>J. Dairy Sci.</i> 65:1493–1502
Lundquist et al. (1983)	<i>J. Dairy Sci.</i> 66:475–491
Ray et al. (1983)	<i>J. Dairy Sci.</i> 66:2084–2092
Huber et al. (1984)	<i>J. Dairy Sci.</i> 67:2525–2531
Lundquist et al. (1985)	<i>J. Dairy Sci.</i> 68:3350–3354
Lundquist et al. (1985)	<i>J. Dairy Sci.</i> 68:3055–3058
Higginbotham et al. (1987)	<i>J. Dairy Sci.</i> 70:630–634
Jones et al. (1988)	<i>J. Dairy Sci.</i> 71:525–529
Hansen et al. (1991)	<i>J. Dairy Sci.</i> 74:1361–1369
Koenig et al. (2002)	<i>J. Dairy Sci.</i> 85:930–938
Phillips et al. (2003)	<i>J. Dairy Sci.</i> 86:3634–3647
Piepenbrink et al. (2004)	<i>J. Dairy Sci.</i> 87:1071–1084
Noftsgger et al. (2005)	<i>J. Dairy Sci.</i> 88:223–237
St-Pierre and Sylvester (2005)	<i>J. Dairy Sci.</i> 88:2487–2497
Rulquin et al. (2006)	<i>J. Dairy Sci.</i> 89:4387–4394
Johnson-VanWieringen et al. (2007)	<i>J. Dairy Sci.</i> 90:5176–5188
Wang et al. (2010)	<i>J. Dairy Sci.</i> 93:3661–3670
Chen et al. (2011)	<i>J. Dairy Sci.</i> 94:1978–1988
Klangnok et al. (2011)	<i>Suranaree J. Sci. Technol.</i> 18:99–108
Plank (2011)	Thesis
Martin et al. (2013)	<i>Anim. Feed Sci. Tech.</i> 182:16–24
Jenkins (2014)	Thesis
Lee et al. (2015)	<i>J. Dairy Sci.</i> 98:1234–1247
Clements (2015)	Thesis
Baldin (2016)	Dissertation
Lopez et al. (2016)	<i>J. Anim. Sci.</i> 94:2485–2496
Sun et al. (2017)	<i>J. Anim. Sci.</i> 88:602–609
Baldin et al. (2018)	<i>J. Dairy Sci.</i> 101:376–385
Tucker et al. (2015)	Novus International report