

# Dietary Molasses Increases Ruminal pH and Enhances Ruminal Biohydrogenation During Milk Fat Depression

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## **INTRODUCTION**

Recent work from our group and others has suggested that dietary sugars have the potential to increase milk fat synthesis by dairy cows in certain situations. Milk fat depression (**MFD**) is caused by an interaction of dietary factors which influence ruminal fermentation and the availability of unsaturated fatty acids (**FA**). Unique FA produced in this rumen environment are capable of altering mammary function to decrease synthesis of milk fat, and results from an initial study at Kansas State University suggested that feeding 5% molasses could decrease the production of these MFD-inducing FA (Bradford and Titgemeyer, 2008). However, in addition to the signs of improved milk fat synthesis in molasses-fed cows, our previous study also showed a decrease in milk protein yield with the 5% molasses treatment.

Our primary aim in the current study was to evaluate ruminal and milk parameters to better understand the mechanisms underlying increased *de novo* FA synthesis in response to dietary molasses. In addition, we assessed production responses with or without postruminal infusion of essential amino acids to determine whether negative effects of molasses on protein yield could be overcome with increased supply of limiting amino acids.

## **MATERIALS AND METHODS**

**Animals and treatments.** Six cannulated, multiparous, late-lactation Holstein cows ( $220 \pm 18$  DIM) were used to evaluate effects of adding molasses, with or without supplemental amino acids, on ruminal parameters and milk composition. The control diet was formulated with the intention of causing MFD, and included 37% forage and 20% corn DDGS, resulting in a diet with 24.5% NDF, 48.7% NFC, and 4.7% ether extract. Molasses was included at 5% of diet DM in the treatment diet, replacing a portion of the corn grain. The cane molasses used in this study was 53% total sugars (DM basis) and 70% DM, and did not include any additives. Composition and nutrient densities for the experimental diets are shown in **Table 1**. A common base mix representing 95% of diet DM was prepared daily, and ground corn grain or molasses was added to complete each TMR. Throughout the experiment, cows were housed in a tie-stall facility, milked three times daily (500 h, 1300 h, and 2100 h), and fed twice daily (630 h and 1500 h) for *ad libitum* intake.

The experiment was designed as a split-plot with dietary treatments tested as the main plot, arranged in a switchback design (3 periods). Abomasal infusion treatments were tested as the

subplot and were arranged as a crossover within each main plot period. Cows were randomly assigned to sequence of treatments. Dietary treatments were fed for 28 d, with 16 d for diet adaptation and the final 12 d for 2 abomasal infusion periods. Abomasal infusions of water or amino acids (5 g/d L-methionine + 15 g/d L-lysine-HCl + 5 g/d L-histidine-HCl-H<sub>2</sub>O) were administered 3 times daily for 5 d, with 2 d between infusion periods.

**Sample and data collection and analysis.** Milk production data and samples were collected during the final 4 d of each infusion period for analysis of milk components and FA profiles. Milk samples were analyzed to determine concentrations of fat, protein, lactose, and urea nitrogen (Heart of America DHIA, Manhattan). Additional milk samples used for FA analysis were thawed, shaken, and composited into 1 sample per cow/subperiod. The composited samples were lyophilized, resuspended in 1 mL of hexane containing C13:0 as an internal standard, and methylated using BF<sub>3</sub>-methanol. The resulting FA methyl esters were extracted in hexane and injected onto a Supelco SP-2560 capillary GC column for FA profile analysis.

Ruminal contents were collected at 9-h intervals on d 26-28 of each dietary period, representing every 3 h of a 24-h period. Grab samples of ruminal digesta were collected from 5 locations throughout the rumen and mixed. A portion of this sample was frozen at -20°C for microbial population analysis, and a second subsample was strained through four layers of cheese cloth to obtain a fluid sample. Rumen fluid pH was measured using a portable pH meter (Orion Research, Boston, MA), and a sample was frozen at -20°C until compositing for analysis of VFA and NH<sub>3</sub>. Ammonia was determined colorimetrically using an autoanalyzer (Technicon Analyzer II, Technicon Industrial Systems, Buffalo Grove, IL). Ruminal VFA were measured using a gas chromatograph (Model 5890, Hewlett-Packard, Avondale, PA).

Samples used for microbial population analysis were thawed at room temperature, composited by cow within period, and homogenized using a high-speed blender. Microbial DNA was then isolated using a commercial kit (ZR Fecal DNA Kit, Zymo Research Corp., Orange, CA). Isolated DNA was quantified by spectroscopy (Nanodrop-1000, Nanodrop Technologies Inc., Wilmington, DE), and sample volumes were adjusted to achieve uniform DNA concentrations across all samples. Quantitative real-time PCR was used to determine relative abundance of bacterial populations using previously validated primers specific for genes encoding 16S ribosomal RNA (Stevenson and Weimer, 2007). Fungi and protozoa were quantified using primers designed for regions of 18S ribosomal RNA and internal transcribed spacer regions (Denman and McSweeney, 2006; Sylvester et al., 2004). The cycle-to-threshold value obtained with a primer pair designed to detect all bacteria was used as the reference value for the other genera- and species-specific assays, and a common fluorescence threshold was used for all species. For each sample, relative abundance of each population was calculated ( $2^{-\Delta CT}$ ).

Data were analyzed using mixed models including the fixed effects of diet, infusion, and their interaction as well as the random effects of period and cow. Ruminal measures were collected only at the end of the dietary periods, so infusion effects and interactions were not tested for these variables. Ruminal microbe population data were log-transformed for statistical analysis and reported means are back-transformed. Significance was declared at  $P < 0.05$ , and tendencies were declared at  $P < 0.10$ .

## **RESULTS AND DISCUSSION**

***Milk protein.*** Increasing dietary molasses linearly decreased milk protein yield in our previous study, which led us to use essential amino acid infusions to test whether molasses created a metabolizable protein limitation. However, in the present experiment, we did not observe any effect of molasses on milk protein content or yield (**Table 2**,  $P > 0.40$ ). Furthermore, amino acids did not increase milk protein content or yield, and no interaction with diet was observed (all  $P > 0.35$ ). Therefore, these results do not support our previous finding that dietary molasses negatively affects milk protein yield during MFD.

In addition to the lack of an effect on milk protein, infusion of amino acids had no significant effect on any other endpoints measured ( $P > 0.10$ ), and no interactions with diet were observed ( $P > 0.15$ ). We interpret this lack of response to suggest that metabolizable protein supply was not likely the primary limitation to milk production for either diet used in this study.

***Productivity and milk fat.*** Consistent with previous findings, molasses had no effect on dry matter intake during MFD (Table 2). Interestingly, molasses tended to decrease BW gain compared to control ( $-3$  vs.  $+16 \pm 9$  kg/21 d,  $P = 0.06$ ). Dietary molasses increased milk fat concentration ( $P < 0.01$ ) with no significant effect on milk yield. However, because of numerical differences in milk yield, molasses did not significantly increase yields of milk fat ( $P = 0.25$ ) or energy-corrected milk ( $P = 0.18$ ). Molasses had no significant effects on content or yield of protein, lactose, or urea N. Combined with the results of our previous study, replacing corn grain with 5% molasses (DM basis) during MFD appears to increase milk fat content by approximately 10%, but increases milk fat yield by a more modest 5%.

Milk FA analysis was used to further probe the mechanisms underlying milk fat responses to dietary molasses. In our previous work, we observed an increase in the yield of FA derived from *de novo* synthesis in the mammary gland, suggesting that molasses was able to relieve the inhibition of this process during MFD. However, we were not able to replicate this observation in the current study; diet had no significant effects on total yields of *de novo* synthesized (short- and medium-chain) or preformed (long-chain) FA (**Table 3**,  $P > 0.32$ ). Individual FA implicated in the biohydrogenation theory of MFD were also assessed. However, the numeric increase in short- and medium-chain FA supports the concept that *de novo* synthesis of FA in the mammary gland might be increased by dietary molasses. Molasses did not significantly alter the concentration or yield of *trans*-10, *cis*-12 CLA, one FA known to induce MFD (Harvatine et al., 2009). However, dietary molasses decreased the yield of *trans*-10 C18:1 and increased the yield of *trans*-11 C18:1 in milk (Table 3,  $P < 0.01$ ). *Trans*-10 C18:1 is nearly always elevated in cases of MFD, and can be used as a marker of ruminal conditions that promote MFD, whereas *trans*-11 C18:1 is produced as an intermediate in the “normal” ruminal biohydrogenation pathway (Harvatine et al., 2009). Therefore, these shifts in FA profile suggest that dietary molasses had a positive influence on biohydrogenation by promoting normal biohydrogenation and decreasing flux through the alternative pathway.

Additionally, molasses decreased the  $\Delta 9$ -desaturase index (quantified as C14:1/C14,  $P < 0.01$ ), consistent with our previous findings. The  $\Delta 9$ -desaturase enzyme is the most important determinant of the relative saturation of milk fat, which has potential implications for the healthfulness of milk in human diets. MFD is typically associated with decreased  $\Delta 9$ -desaturase

activity, and decoupled effects on milk fat yield and  $\Delta 9$ -desaturase activity suggest that molasses may increase the production of a FA that inhibits  $\Delta 9$ -desaturase activity without greatly decreasing milk fat synthesis pathways in the mammary gland, as several CLA isomers have been shown to do (Harvatine et al., 2009).

***Ruminal metabolism and microbial populations.*** The key addition to the current study was the ability to collect ruminal digesta, allowing us to assess measures of ruminal fermentation. Dietary molasses significantly increased ruminal pH and decreased total VFA concentration in the rumen (**Table 4**,  $P < 0.02$ ). Molasses also increased the molar proportions of acetate and butyrate and decreased the proportion of propionate in ruminal fluid, although these effects were not dramatic (Table 4). We also analyzed rumen contents to quantify the relative abundance of key microbial populations, but were unable to detect any treatment differences (**Table 5**). The lack of effects on these populations despite changes in measures of ruminal fermentation could simply mean that cow-to-cow variation in populations (or random variation over time) is too great to allow for conclusive demonstration of treatment effects; for example, we observed 2-fold numerical differences between treatments in protozoa, but this did not achieve statistical significance. In addition, molasses may have effects on metabolism by altering metabolic activity of various populations more so than by altering the abundance of the specific populations. Finally, it must be pointed out that all of the individual bacterial species quantified in this analysis represent less than 6% of all bacteria in the rumen, making the point that techniques available for such analysis are far from comprehensive at this time.

The observed effects of molasses on measures of ruminal metabolism are consistent with responses to dietary sucrose in a recent report (Penner and Oba, 2009). Penner and Oba fed early-lactation cows diets including 0% or 4.7% sucrose, and found that sucrose tended to increase mean ruminal pH, decreased the proportion of *trans*-C18:1 FA in milk, and tended to increase milk fat yield by 7%. Given that sugar sources are fermented very rapidly in the rumen, the observed positive effects on rumen pH are somewhat surprising. However, ruminal pH is influenced not only by acid production, but also by buffering and acid removal. One possible explanation for this response is that VFA uptake from the rumen is stimulated by molasses. Molasses generally increases butyrate production, and butyrate stimulates blood flow to the ruminal epithelium to a greater extent than other VFA (Sellers et al., 1964; Thorlacius, 1972). Increased blood flow to the epithelium would result in more rapid transfer of VFA from epithelial cells to the bloodstream, in turn creating an increased gradient between the rumen and the epithelial cells. This potential increase in uptake rate could explain the decrease in ruminal VFA concentration and increased ruminal pH in the molasses treatment. However, numerous other mechanisms could also explain the response (Penner and Oba, 2009).

Regardless of the mechanism underlying the improved ruminal pH in cows fed sugar sources, decreased acid load provides a logical explanation for the improvement in biohydrogenation. Fiber-digesting bacteria are thought to be primarily responsible for ruminal biohydrogenation of FA (Harfoot and Hazlewood, 1988), and these bacteria are especially susceptible to growth inhibition by low pH. Taken as a whole, recent data suggests that in a MFD-environment, molasses increases ruminal pH, promoting maintenance of normal biohydrogenation and recovery of milk fat yield by 5 – 10%.

## **CONCLUSIONS**

Replacing 5% of dietary corn with cane molasses (DM basis) in a low-forage, high-concentrate diet increased ruminal pH, altered ruminal biohydrogenation to decrease production of intermediates associated with MFD, and increased milk fat content. However, no significant effects were detected for milk component yield. Nevertheless, considered along with other recently published data, dietary molasses appears to mildly increase milk fat yield by promoting normal ruminal biohydrogenation and decreasing absorption of MFD-inducing FA.

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Table 1. Ingredient and Nutrient Composition of Diets<sup>1</sup>

	Dietary Molasses	
	0%	5%
<b>Ingredient</b>		
Corn silage	25.5	25.5
Alfalfa hay	12.6	12.6
Corn DDGS	19.8	19.8
Ground corn grain	33.6	28.8
Molasses	-	4.8
Soybean meal	4.1	4.1
Expeller soybean meal	2.7	2.7
Limestone	1.1	1.1
Trace mineral salt	0.4	0.4
Micronutrient premix	0.2	0.2
<b>Nutrient</b>		
Dry matter	66.4	65.8
Crude protein	16.2	16.1
Neutral detergent fiber	24.5	24.5
Non-fiber carbohydrate	48.7	48.6
Starch	36.3	32.9
Sugars (by invertase)	6.4	8.9
Ether extract	4.7	4.6
Ash	5.9	6.2

<sup>1</sup> Values other than dry matter are expressed as a percentage of diet dry matter.

Table 2. Effects of Treatment on Productivity of Lactating Dairy Cows

Infusion: Dietary molasses:	Water		Amino acids		SEM	<i>P</i> value <sup>1</sup>	
	0%	5%	0%	5%		Molasses	AA
Dry matter intake, kg/d	24.5	24.4	23.9	24.6	1.3	0.41	0.66
Milk yield, kg/d	28.3	28.6	29.0	28.1	2.8	0.70	0.86
SCM, kg/d	24.2	24.9	25.0	24.8	2.6	0.67	0.53
ECM, kg/d	26.6	27.3	27.5	27.3	2.8	0.18	0.45
Milk fat, %	2.68	<b>2.90</b>	2.75	<b>2.98</b>	0.21	<b>0.01</b>	0.23
Milk protein, %	3.45	3.37	3.44	3.44	0.13	0.41	0.37
Milk lactose, %	4.91	4.92	4.93	4.90	0.08	0.74	0.99
Milk fat, kg/d	0.79	0.83	0.81	0.85	0.11	0.25	0.49
Milk protein, kg/d	0.97	0.95	0.98	0.96	0.09	0.43	0.73
Milk lactose, kg/d	1.41	1.42	1.42	1.40	0.14	0.81	0.89
Milk urea N, mg/dL	11.6	11.2	11.4	11.3	0.7	0.30	0.97

<sup>1</sup> Contrasts: Molasses = effect of dietary molasses; AA = effect of amino acid infusion. All molasses x AA effects were non-significant for reported variables ( $P > 0.15$ ).

Table 3. Effects of Treatment on Yield of Selected Milk Fatty Acids

Infusion: Dietary molasses:	Water		Amino acids		SEM	<i>P</i> value <sup>1</sup>	
	0%	5%	0%	5%		Molasses	AA
Fatty acid, g/d							
<i>trans</i> -10 C18:1	16.8	<b>10.9</b>	12.8	<b>10.4</b>	3.3	<b>0.01</b>	0.15
<i>trans</i> -11 C18:1	12.2	<b>14.7</b>	12.3	<b>14.1</b>	3.0	<b>&lt; 0.01</b>	0.76
Total <i>trans</i> C18:1	32.3	28.7	27.8	27.8	3.0	0.28	0.13
<i>trans</i> -10, <i>cis</i> -12 CLA	0.19	0.18	0.18	0.15	0.03	0.37	0.42
Total unsaturated	268	261	250	255	29	0.92	0.24
Short- and medium- chain (< C16)	152	162	144	150	26	0.33	0.23
C16	183	188	169	177	21	0.43	0.14
Long-chain (> C16)	325	323	309	318	41	0.76	0.42
Desaturase index (C14:1/C14)	0.13	<b>0.11</b>	0.12	<b>0.11</b>	0.02	<b>&lt; 0.01</b>	0.28

<sup>1</sup> Contrasts: Molasses = effect of dietary molasses; AA = effect of amino acid infusion. All molasses x AA effects were non-significant for reported variables ( $P > 0.15$ ).

Table 4. Effects of Molasses Inclusion Rate on Measures of Ruminal Fermentation

	Dietary molasses		SEM	P value
	0%	5%		
Ruminal pH	5.73	5.87	0.06	<b>0.02</b>
Total VFA, mM	140.8	132.7	4.6	< <b>0.01</b>
Acetate, mol / 100 mol	46.3	46.9	0.9	<b>0.04</b>
Propionate, mol / 100 mol	28.7	27.4	1.4	<b>0.01</b>
Butyrate, mol / 100 mol	16.7	17.7	1.0	<b>0.04</b>
Valerate, mol / 100 mol	4.9	4.9	0.3	0.78
Ammonia, mg/dL	7.86	7.35	0.88	0.32

Table 5. Effects of Molasses Inclusion Rate on Relative Abundance of Selected Ruminal Microbe Populations

% of total bacteria	Dietary molasses		SEM	P value
	0%	5%		
<i>Butyrivibrio fibrisolvens</i> group	0.00068	0.00102	0.00074	0.60
<i>Eubacterium ruminantium</i>	0.164	0.127	0.043	0.52
<i>Fibrobacter succinogenes</i>	1.17	0.84	0.39	0.60
<i>Megasphaera elsdenii</i>	0.011	0.011	0.012	0.93
<i>Prevotella bryantii</i>	0.78	0.43	0.31	0.29
<i>Prevotella ruminicola</i>	1.39	1.21	0.50	0.76
<i>Prevotella</i> (genus)	85.6	50.1	18.5	0.29
<i>Ruminococcus flavefaciens</i> group	1.58	0.85	0.50	0.26
<i>Streptococcus bovis</i> group	0.0057	0.0050	0.0025	0.83
Protozoa	5.28	2.01	2.20	0.14
Fungi	0.21	0.12	0.19	0.47