Effects of supplemental calcium gluconate embedded in a hydrogenated fat matrix on lactation, digestive, and metabolic variables in dairy cattle

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ABSTRACT

There is growing evidence suggesting that by improving gut integrity and function, less energy is partitioned toward immune responses related to xenobiotic infiltration, sparing energy for productive purposes. Gluconic acid and its salts have previously shown prebiotic effects in the lower gut of nonruminant animals, where they serve as a precursor for butyrate, although evidence in ruminants is limited. Butyrate and its fermentative precursors have demonstrated multiple beneficial effects to gastrointestinal ecology, morphology, and function, such as the stimulation of epithelial cell proliferation and improvement of gut barrier function and ecology. The objective of this study was to evaluate changes in milk production, milk fatty acid composition, and fecal and blood parameters in lactating dairy cattle fed a hydrogenated fat-embedded calcium gluconate (HFCG) supplement designed to target the hindgut for calcium gluconate delivery. In addition, the effects of a compound feed processing method (i.e., incorporated into a mash or an extruded pellet) were tested to evaluate the effect of extrusion on product efficacy. Forty-five lactating Holstein cows at approximately 165 d in milk were used in a 3×3 Latin square consisting of three 28-d periods, during which animals were offered a basal ration mixed with 3 different compound feeds: a negative control in mash form containing no HFCG, or the HFCG supplement fed at a target rate of 16 g/d, delivered in either a mash or pelleted form. Supplementation of HFCG tended to increase yields of milk fat and fat- and energy-corrected milk. Total yields and concentrations of milk fatty acids ≥ 18 carbons in length tended to increase in response to HFCG. Plasma nonesterified fatty acids and milk urea increased in HFCG treatments. No differences were observed in fecal pH or fecal concentrations of volatile fatty acids, with the exception of iso-

butyrate, which decreased in HFCG-fed cows. Changes in milk fatty acid profile suggest that increased milk fat yield was driven by increased incorporation of pre-formed fatty acids, supported by increased circulating nonesterified fatty acid. Future research investigating the mode of action of HFCG at the level of the hindgut epithelium is warranted, as measured fecal parameters showed no response to treatment.

Key words: hindgut, prebiotic, milk fat

INTRODUCTION

The gastrointestinal tract is highly sensitive to a plethora of factors intrinsic to the lifecycle of dairy cows. As recently reviewed by Sanz-Fernandez et al. (2020), the hindgut is susceptible to acidosis, much like the rumen; however, due to a variety of physiological and structural differences, it may be less capable of tolerating an acidotic challenge than the rumen, resulting in increased permeability to xenobiotic compounds and the repartitioning of energy to support an immune response (Fox et al., 2005; Kvidera et al., 2017). In human and nonruminant models, eubiotic supplementation is an extensive practice aimed at promoting microbiome stability in the large intestine. Gluconic acid is a mild organic acid 6 carbons in length with an acid dissociation constant (pK_a) of 3.7, produced by the dehydrogenation of glucose by various fermentative microorganisms (Ramachandran et al., 2006), which acts as a prebiotic in nonruminant species by passing through the stomach and small intestine with minimal digestion and absorption, allowing it to be fermented by microbes in the lower gut, and resulting in improved production performance (Biagi et al., 2006; Poeikhampha and Bunchasak, 2011; Michiels et al., 2020). This association between prebiotic supply and animal performance is thought to be mediated by changes in microbial VFA production (Tsukahara et al., 2002, 2006; Biagi et al., 2006).

The beneficial effects of VFA on intestinal health are broadly accepted. Increased VFA supply (Velazquez et al., 1997; Hamer et al., 2008), and in particular increased butyrate (Sakata and Tamate, 1978; Gálfi et al., 1991;

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Mentschel et al., 2001), induces epithelial cell proliferation in the gut in ruminant and nonruminant models. Butyrate supports homeostasis in the gastrointestinal tissues by modulating the balance between proliferation, apoptosis, and differentiation, while stimulating increases in local blood flow (Kristensen and Harmon, 2004; Hamer et al., 2008). When butyrate levels are clinically low, supplementation with butyryl esters has proven to be an effective therapy, particularly in individuals with chronic inflammation and loss of function in the absorptive surfaces of the gut (Wachtershauser and Stein, 2000; Miyoshi et al., 2011; Leonel et al., 2013). The effects of ruminal butyrate supplementation are well documented (Huhtanen et al., 1993; Miettinen and Huhtanen, 1996; Herrick et al., 2018), as are the effects of butyrate in nonruminant species (Biagi et al., 2006; Poeikhampha and Bunchasak, 2011; Michiels et al., 2020). However, there is a lack of information on the effects of butyrate in the lower gut of ruminants, more specifically the lactating dairy cow, and the exact mode of action of butyrate in the hindgut is also not well defined (as reviewed by den Besten et al., 2013; Liu et al., 2018).

Given the performance responses reported in non-ruminant applications, there are potential benefits to providing hydrogenated fat-embedded gluconic acid in the mature ruminant. Postruminal infusion of calcium gluconate, a gluconic acid salt, has resulted in changes in fecal VFA profile and improved milk fat concentration in lactating dairy cows (Doelman et al., 2019b; McKnight et al., 2019). In a recent pilot study conducted by our group, a hydrogenated fat-embedded calcium gluconate (**HFCG**) compound fed to early-lactation dairy cows increased milk fat concentration and yield (Doelman et al., 2019a), in contrast to the null response observed when incorporating calcium gluconate directly into the diet without embedding it in fat (McKnight et al., 2019). This suggests that the hydrogenated fat matrix offers some degree of protection to the calcium gluconate from degradation in the rumen (Papas and Wu, 1997; Wu et al., 2012), and that following digestion of the fat matrix in the small intestine, the active ingredient is liberated and available for fermentation in the hindgut. A recent in sacco study conducted by our group (unpublished) showed 75 to 80% retention of calcium gluconate when HFCG was incubated for 6 h. This suggests that although a portion of the calcium gluconate in the product may be able to solubilize in the rumen environment or be available for catabolism by rumen microbiota, the majority is able to remain intact.

The objective of the current study was to test the efficacy of supplementing calcium gluconate embedded in a hydrogenated fat matrix on milk production, milk

fatty acid profile, fecal VFA composition, and circulating metabolites in lactating dairy cows. Additionally, 2 methods of delivery of the calcium gluconate supplement (incorporated into either a mash or pellet compound feed) were tested to assess any effects of feed processing, specifically any changes due to pelleting at high temperatures. We hypothesized that supplementation with the HFCG product would result in increased milk fat concentration and yield relative to the control group, with minimal differences between delivery methods.

MATERIALS AND METHODS

This study was conducted at the Trouw Nutrition Dairy Research Facility (Boxmeer, the Netherlands) between April and June 2016. The study complied with the Dutch Act on Animal Experiments, which complies to European Directive 2010/63/EU, and was approved by the competent authority, the Centrale Commissie Dierproeven.

Animal Feeding and Management

For the duration of the study, animals were group housed in a freestall barn. Both feed and water were provided *ad libitum*. Feed was delivered as a TMR daily at approximately 1000 h, and individual daily feed intakes were monitored using electronic feed bunks (RIC system; Hokofarm Group B.V.). The basal ration (Table 1) consisted of corn silage, grass silage, grass hay, and a compound feed and was formulated to provide an estimated 7.01 MJ of NE_L /kg of DM and 15.4% CP. Samples of the basal ration were collected 3 times per week (~500 g/sample) and stored at -20°C until analysis. At that time, samples were thawed, pooled by week, and analyzed in triplicate for DM content as described by Seymour et al. (2019). Proximate analyses were conducted using wet chemistry by MasterLab (Boxmeer, the Netherlands).

Experimental Design

Based on milk fat responses from previous studies (Doelman et al., 2019a,b; McKnight et al., 2019), a sample size calculation was performed to determine the number of animals required to detect a 0.17% difference in milk fat concentration, given a herd average milk fat concentration of 4.20%, standard deviation (**SD**) of 0.30%, and type I error rate of 5%. Based on these calculations, 45 lactating Holstein-Friesian dairy cattle (165 ± 53 DIM at the beginning of the study, mean $\pm$ SD) were enrolled in a study utilizing a 3×3 replicated Latin square design consisting of 3 consecutive 28-d

Table 1. Formulation and chemical composition of basal ration¹

Item	Content
Ingredient	
Corn silage	41.4
Compound feed ²	32.8
Grass silage	20.4
Grass hay	5.4
Composition	
DM (%)	49.2
NE _L (MJ/kg of DM)	7.01
CP	15.4
Starch	25.0
NDF	32.3
ADF	17.8
ADL	1.86
Crude fat ³	3.28
Ash	8.58
Ca	0.58
P	0.37
Na	0.17
K	1.60
Vitamin A (IU/kg of DM)	2,996
Vitamin D (IU/kg of DM)	749
Vitamin E (IU/kg of DM)	20.0

¹Values are presented as a percentage of DM unless stated otherwise.

²Contained (as-fed %) corn (38.19), 48% CP soybean meal (16.84), rumen-protected rapeseed meal (10.00), solvent-extracted rapeseed meal (10.00), wheat (7.74), soy hulls (5.64), cane molasses (5.00), rumen-protected soybean meal (1.71), limestone (1.59), urea (1.50), vitamin/mineral premix (1.40), and vitamin E (0.02).

³Addition of hydrogenated fat-embedded calcium gluconate increased crude fat to approximately 3.31% DM.

periods. Each period consisted of 24 d of adaptation followed by 4 d of data and sample collection. Multiparous cows ($n = 31$) were blocked by parity and average daily yields of milk, fat, and protein during the previous lactation. Primiparous heifers ($n = 14$) with no previous production records were blocked by expected production based on genetic evaluation. Treatment sequences were randomly assigned to each animal within a block by the study investigators. Treatments consisted of a negative control mash (e.g., no HFCG supplementation) and 2 delivery forms of the HFCG supplement (Trouw Nutrition), one being a mash application, and the other a pellet extruded at $80 \pm 3^\circ\text{C}$. The HFCG supplement (50% hydrogenated palm fat, 40% calcium gluconate, 10% calcium carbonate) was incorporated into the compound feed at an inclusion rate of 1.93 kg/t to achieve a targeted inclusion of 0.07% DM, corresponding to approximately 16 g of supplement per day, providing 6.40 g of calcium gluconate at the targeted intake of 22.31 kg of DM per day. Because treatments were administered in different physical forms, no blinding was used. All animals started the experiment on the same day and were acclimated to the negative control ration for 2 wk before the start of the first period. After exiting the milking parlor, animals were weighed daily,

using an inline scale, and the average BW from d 25 to 28 of each period was evaluated.

Blood and Fecal Analyses

Blood samples were collected at 0800 and 1300 h on d 28 from the coccygeal vessels into 10-mL sodium heparin and potassium EDTA blood collection tubes (Becton, Dickinson and Co.). Samples were immediately stored on ice until centrifugation at 4°C at $1,500 \times g$ for 15 min, after which plasma was aliquoted into polypropylene vials. Plasma samples collected at 0800 h were stored at 4°C until the collection of samples at 1300 h, after which plasma aliquots from both time points were pooled by preservative type and stored at -20°C until analysis by MasterLab (Boxmeer, the Netherlands). Spectrophotometric assays were used to determine the plasma concentrations of acetic acid (K-ACETRM kit, Megazyme), glucose (Glucose GO assay kit, Sigma-Aldrich; Raabo and Terkildsen, 1960), nonesterified fatty acids (NEFA; NEFA-HR(2) kit, Fujifilm Wako Chemicals Europe GmbH; Johnson and Peters, 1993), BHB (Cant et al., 1993), triacylglycerol (Weekes et al., 2006), insulin (Rat Insulin ELISA Kit, Crystal Chem Inc.; Nichols et al., 2016), cholesterol (Cholesterol Quantitation Kit, Sigma-Aldrich), and growth hormone (Weber et al., 2007).

At 1300 h on d 28 of each period, approximately 150 to 200 g of fresh feces was collected directly from the rectum for the determination of DM content. A second sample of approximately 5 g was collected for the determination of VFA composition. Both samples were stored on ice in sealed plastic bags until analysis. Dry matter content was determined by drying in an oven at 60°C for 48 h. For determination of VFA content, 5 g of fresh feces was dissolved in 10 mL of water and mixed thoroughly, and the pH was measured using a glass electrode. An aliquot of approximately 6 mL was centrifuged at $2,700 \times g$ at 10°C for 15 min, and a 1-mL aliquot of the supernatant was subsequently mixed with 0.5 mL of 0.02 M sulfuric acid stored at -20°C until VFA analysis by HPLC.

Milk Samples

Animals were milked twice daily at 0500 and 1700 h. Milk yields were recorded automatically at each milking. Milk was sampled on Tuesday and Thursday mornings and Monday and Wednesday evenings between d 25 and 28 of each experimental period. Samples were collected in tubes containing sodium azide and bronopol as preservatives and stored at 4°C . Samples were pooled within animal by weighing each individual

sample proportionally to yield per milking. Samples were analyzed for fat, CP, lactose, and urea concentration (Qlip, Deventer, the Netherlands) using a Foss Milkscan FT6000 (Foss; ISO, 2013). Yields of ECM were calculated as $[0.01 \times \text{milk yield (kg/d)} + 12.2 \times \text{fat yield (kg/d)} + 7.7 \times \text{protein yield (kg/d)} + 5.3 \times \text{lactose yield (kg/d)}]$; Sjaunja et al., 1990], whereas 4% FCM yields were calculated as $[0.4 \times \text{milk yield (kg/d)} + 15 \times \text{fat yield (kg/d)}]$; Gaines, 1928].

Additional milk samples were collected for the determination of milk fatty acid content where samples were pooled within animal by weighing each individual sample proportionally to yield per milking. After blocking and treatment allocation, cows within a treatment were randomly assigned to 1 of 3 groups consisting of 5 cows per group. There were 9 groups across the total of 45 cows used in this experiment, and cows remained in their groups for the duration of the experiment. A 1-mL sample of milk from each animal was pooled within treatment group, resulting in a 5-mL sample per treatment group per period. Pooled samples were stored at -20°C until analysis by GC-MS at Qlip. Milk fatty acid content of pooled samples was considered representative of the average fatty acid content of milk from animals within treatment groups within periods. Milk fatty acid yields of individual animals were estimated by multiplying the individual milk fat yield of an animal within a period by the milk fatty acid content of the corresponding pooled sample.

Statistical Analyses

All statistical analyses were conducted using the GLIMMIX procedure of SAS v9.4 (SAS Institute Inc., Cary, NC). For data pertaining to animal performance,

blood metabolites and fecal VFA content, the statistical model used was

$$y_{ijklm} = \mu + trt_i + block_j + period_k (block_j) + cow_l (block_j) + parity_m + \varepsilon_{ijklm},$$

where y is the response variable, μ is the overall mean, and ε_{ijklm} is the residual error. The effects of treatment (trt_i ; $i = 1-3$), period within block ($period_k$; $k = 1-3$), and parity group ($parity_m$; $m = 1, 2$, primiparous or multiparous) were considered fixed, whereas the effects of block ($block_j$; $j = 1-15$) and animal within block (cow_l ; $l = 1-3$) were considered random. Because samples for the determination of milk fatty acid content were pooled by treatment group within period, these data were analyzed with a model considering treatment and period as fixed effects, with sample pooling group as a random effect. In all analyses, the Kenward-Roger correction was used to adjust the denominator degrees of freedom, and orthogonal contrasts were used to perform specific hypothesis tests comparing the pooled effect of HFCG supplementation relative to control, and the effect of processing method. Significance was declared where $P < 0.05$, and tendencies where $0.05 \leq P < 0.15$.

RESULTS

Fecal VFA Content and pH

Fecal VFA content and pH are presented in Table 2. No significant differences were observed between treatment groups for pH or concentrations of acetate, propionate, or butyrate. Isobutyrate concentrations decreased ($\sim 15\%$; $P \leq 0.001$) in response to HFCG

Table 2. Fecal pH, DM, and VFA content in response to supplementation with fat-embedded calcium gluconate (HFCG) in either a mash or pelleted form ($n = 45$)

Item	Supplement			SED ¹	P-value ²	
	Control	Mash	Pellet		HFCG	Process
pH	6.55	6.54	6.56	0.031	0.990	0.421
DM (%)	13.0	13.0	13.0	0.27	0.929	0.992
VFA ($\mu\text{mol/g}$)						
Acetate	522	522	505	19.3	0.625	0.378
Propionate	137	134	131	5.1	0.287	0.608
Butyrate	85.7	85.9	88.1	5.35	0.778	0.689
Isobutyrate	17.1	15.6	13.6	0.68	<0.001	0.004
VFA ($\mu\text{mol/g}$ of DM)						
Acetate	4,090	4,070	3,950	172	0.598	0.473
Propionate	1,060	1,030	1,020	43.1	0.270	0.842
Butyrate	654	657	687	42.1	0.714	0.613
Isobutyrate	133	120	106	6.0	0.001	0.017

¹Standard error of the difference.

²HFCG = pooled calcium gluconate effect; Process = mash versus pellet effect.

Table 3. Plasma metabolite concentrations in response to supplementation with fat-embedded calcium gluconate (HFCG) in a mash or pelleted form (n = 45)¹

Item	Supplement			SED ²	P-value ³	
	Control	Mash	Pellet		HFCG	Process
Glucose	3.69	3.67	3.76	0.043	0.551	0.033
Nonesterified fatty acids	0.065	0.085	0.097	0.0098	0.003	0.225
BHB	0.766	0.757	0.708	0.0239	0.116	0.046
Triacylglycerol	0.087	0.086	0.086	0.0024	0.559	0.927
Cholesterol	4.70	4.80	4.77	0.071	0.180	0.731
Insulin (mIU/L)	16.1	16.2	15.6	0.73	0.730	0.477
Somatotropin (ng/mL)	2.33	2.18	2.35	0.111	0.509	0.126

¹Means are presented in mmol/L unless stated otherwise.²Standard error of the difference.³HFCG = pooled calcium gluconate effect; Process = mash versus pellet effect.

supplementation and were lower in the pelleted form relative to the mash (Table 2).

Plasma Metabolites

Plasma metabolite concentrations are presented in Table 3. Concentrations of NEFA increased (40.0%; $P = 0.003$), whereas BHB tended to decrease (4.4%; $P = 0.116$) in response to HFCG supplementation. In addition, BHB was 6.5% higher ($P = 0.046$) in response to the mash relative to the pellet. Concentrations of glucose were elevated ($P = 0.033$) in response to the pellet relative to the mash, whereas concentrations of somatotropin tended to be elevated ($P = 0.126$; Table 3).

Production Responses

Overall production responses are presented in Table 4. Milk fat yield tended to increase by approximately 35 g/d (2.4%; $P = 0.062$) in response to HFCG supplementation, resulting in subsequent increases in yields of FCM (2.1%; $P = 0.056$) and ECM (1.8%; $P = 0.086$). Supplementation of HFCG increased milk urea (7.4%; $P = 0.001$) and tended to decrease milk protein content (0.02%; $P = 0.112$; Table 4). The pelleted form of the HFCG supplement increased ($P = 0.020$) lactose content by 0.03 percentage units relative to the mash. Milk fat content did not differ between treatments (Table 4). No effect of HFCG supplementation was observed on BW (Table 4), whereas animals receiving the mash tended to be 4 kg heavier ($P = 0.146$) than animals receiving the pelleted form.

Milk Fatty Acid Profile

Concentrations of milk fatty acids grouped by carbon number are presented in Table 5, with differences mainly detected at the tendency threshold. Concentra-

tions of individual fatty acid isomers are presented in Supplemental Table S1 (<http://dx.doi.org/10.17632/kptvz5k65d.1>). Addition of HFCG stimulated a decrease ($P \leq 0.110$) in the concentration of fatty acids ranging from 8 to 14 carbons in length, whereas concentrations of butyrate (C4:0; Figure 1), total C18 and n-3 fatty acids tended to increase ($P \leq 0.147$). Concentrations of C16 and SFA decreased ($P \leq 0.042$), whereas concentrations of C22:5 *cis*-7,10,13,16,19, total UFA, n-6, MUFA, and PUFA tended to increase ($P \leq 0.078$) in the pelleted treatment relative to the mash. Responses in fatty acid content grouped by source [e.g., de novo, mixed (C16), and preformed] are presented in Figure 1, and highlight a trend for increased preformed fatty acid content (2.08%; $P = 0.073$) in response to the pooled effect of HFCG supplementation.

Average yield of milk fatty acids grouped by carbon number are presented in Table 6, with average yields of individual fatty acid isomers presented in Supplemental Table S2 (<http://dx.doi.org/10.17632/kptvz5k65d.1>). Yields of butyrate, total C17 and C18, C22:5 *cis*-7,10,13,16,19, CLA, total UFA, n-3 and n-6, MUFA, and PUFA increased ($P \leq 0.142$) in response to HFCG supplementation. The yield of total C16 tended to be lower (3.2%; $P = 0.103$) in the pelleted treatment relative to the mash. When comparing the fatty acid groups, the yield of preformed fatty acids tended to be increased by 30.9 g/d (3.9%; $P = 0.099$) in response to HFCG supplementation (Figure 1).

DISCUSSION

Supplementing HFCG tended to increase milk fat yield, and subsequently FCM and ECM yields. This is in agreement with our previous findings, where infusing unprotected calcium gluconate postruminally resulted in increased concentration and average yield of milk fat, as well as increased ECM yield (Doelman et al., 2019b). In contrast, incorporating unprotected calcium

Table 4. Dry matter intake, milk production and composition, and BW in response to supplementation with fat-embedded calcium gluconate (HFCEG) in a mash or pelleted form (n = 45)¹

Item	Control	Supplement		SED ²	P-value ³	
		Mash	Pellet		HFCEG	Process
DMI	23.8	23.9	23.6	0.22	0.957	0.174
Milk yield	31.1	31.5	31.6	0.36	0.155	0.775
FCM yield ⁴	32.0	32.6	32.6	0.40	0.056	0.903
ECM yield ⁵	32.4	32.9	33.0	0.40	0.086	0.915
Gross feed efficiency (kg/kg) ⁶	1.38	1.39	1.41	0.020	0.218	0.207
Fat yield	1.31	1.34	1.34	0.020	0.061	0.766
Protein yield	1.12	1.12	1.13	0.015	0.495	0.744
Lactose yield	1.43	1.44	1.46	0.018	0.117	0.440
Fat content (%)	4.25	4.31	4.27	0.051	0.348	0.465
Protein content (%)	3.62	3.59	3.60	0.018	0.112	0.636
Lactose content (%)	4.59	4.59	4.62	0.012	0.133	0.020
Milk urea (mg/dL)	27.3	29.4	29.2	0.69	0.001	0.837
BW (kg)	571	574	570	2.7	0.683	0.146

¹Means are expressed in kg/d unless stated otherwise.²Standard error of the difference.³HFCEG = pooled calcium gluconate effect; Process = mash versus pellet.⁴4% FCM yield: $0.4 \times \text{milk yield (kg/d)} + 15 \times \text{fat yield (kg/d)}$.⁵ECM yield: $0.01 \times \text{milk yield (kg/d)} + 12.2 \times \text{fat yield (kg/d)} + 7.7 \times \text{protein yield (kg/d)} + 5.3 \times \text{lactose yield (kg/d)}$.⁶ECM yield (kg/d):DMI (kg/d).

gluconate directly into the diet did not improve performance (McKnight et al., 2019). As gluconic acids salts have previously been shown to have low absorption in the small intestine (Asano et al., 1994), the present results suggest that digestion of the fat matrix in the small intestine liberated calcium gluconate, and that

the observed responses in production are derived from the postprandial fermentation of calcium gluconate in the hindgut.

In addition to the overall change in milk fat content, the fatty acid profile was altered with HFCEG supplementation. Fatty acids up to 14 carbons in length

Table 5. Milk fatty acid content in response to supplementation with fat-embedded calcium gluconate (HFCEG) in a mash or pelleted form (n = 9)

Fatty acid content (%)	Control	Supplement		SED ¹	P-value ²	
		Mash	Pellet		HFCEG	Process
C4	3.47	3.58	3.54	0.066	0.147	0.554
C6	2.40	2.40	2.38	0.022	0.651	0.409
C8	1.42	1.39	1.38	0.020	0.110	0.697
C10	3.83	3.9	3.65	0.090	0.064	0.661
C11	0.075	0.067	0.067	0.0056	0.105	0.969
C12	4.29	4.10	4.09	0.097	0.036	0.876
C13	0.118	0.108	0.109	0.0071	0.151	0.817
C14	13.6	13.4	13.4	0.13	0.035	0.855
C15	1.76	1.72	1.72	0.024	0.096	0.769
C16	34.6	34.7	34.1	0.24	0.327	0.042
C17	1.31	1.33	1.35	0.020	0.213	0.312
C18	31.5	31.9	32.2	0.39	0.113	0.370
C20	0.351	0.356	0.348	0.0074	0.839	0.274
SFA	72.6	72.4	71.7	0.33	0.070	0.036
UFA	27.4	27.6	28.3	0.33	0.070	0.036
CLA	0.383	0.387	0.401	0.0114	0.293	0.212
n-3	0.400	0.420	0.414	0.0110	0.079	0.588
n-6	1.72	1.74	1.77	0.014	0.006	0.056
MUFA	23.9	23.9	24.7	0.31	0.154	0.023
PUFA	2.58	2.63	2.70	0.031	0.011	0.050
Unknown fatty acids	0.95	1.08	0.98	0.084	0.302	0.286

¹Standard error of the difference.²HFCEG = pooled calcium gluconate effect; Process = mash versus pellet.

Table 6. Average milk fatty acid yield in response to supplementation with fat-embedded calcium gluconate (HFCG) in a mash or pelleted form (n = 45)

Fatty acid yield (g/d)	Supplement			SED ¹	P-value ²	
	CON	Mash	Pellet		HFCG	Process
C4	45.2	47.9	46.6	1.01	0.032	0.256
C6	31.2	32.1	31.3	0.50	0.266	0.169
C8	18.5	18.6	18.3	0.40	0.942	0.376
C10	49.9	49.4	49.4	1.15	0.655	0.995
C11	0.98	0.90	0.89	0.075	0.256	0.945
C12	55.9	55.0	54.0	1.60	0.320	0.538
C13	1.53	1.44	1.45	0.093	0.294	0.950
C14	177	179	174	3.8	0.782	0.221
C15	22.9	23.1	22.6	0.44	0.928	0.349
C16	451	464	449	8.3	0.403	0.103
C17	17.1	17.7	17.8	0.50	0.137	0.849
C18	410	425	426	11.7	0.142	0.978
C20	4.56	4.76	4.57	0.143	0.429	0.220
SFA	946	969	944	16.1	0.455	0.140
UFA	357	368	373	8.6	0.083	0.564
CLA	4.98	5.14	5.26	0.150	0.121	0.451
n-3	5.21	5.60	5.47	0.193	0.062	0.493
n-6	22.3	23.2	23.3	0.52	0.056	0.881
MUFA	311	318	325	7.9	0.131	0.443
PUFA	33.6	35.1	35.5	0.76	0.025	0.594
Unknown fatty acids	12.4	14.4	12.9	1.07	0.193	0.163

¹Standard error of the difference.²HFCG = pooled calcium gluconate effect; Process = mash versus pellet. **Figure 1.** Milk butyrate (C4:0), de novo fatty acids (even-chain C4:0–C14), total C16, and preformed fatty acids (C18+) responses to the pooled effect of hydrogenated fat-embedded calcium gluconate supplementation, relative to control. Gray bars represent percent change in milk fatty acid content, whereas white bars represent percent changes in milk fatty acid yield. Significance ($P < 0.05$) is indicated with an asterisk, and tendencies ($0.05 \leq P < 0.15$) with a dagger. Error bars indicate SE of the difference as a percentage of the control mean.

are formed almost exclusively via de novo synthesis, whereas those $\geq$ C18 are derived solely from extramammary sources, such as body reserves or diet; C16 fatty acids can be either de novo or extramammary in origin (Grummer, 1991; Garnsworthy et al., 2006). The changes in milk fatty acid content in response to HFCG (Table 5, Figure 1) suggest an overall shift from the de novo synthesis of fatty acids (decreased concentrations of C8 to C14 fatty acids) to increased incorporation of preformed fatty acids, which is reflected in the increased yield of fatty acids $\geq$ 18 carbons in length. This hypothesis is further supported by the increase in plasma NEFA, which would serve to provide the additional substrates necessary to facilitate the increased incorporation of preformed fatty acids. It is possible that a portion of these preformed fatty acids were derived from dietary sources. As no differences in DMI were observed (Table 4), this would require changes in fat digestibility, and given that HFCG likely acts in the hindgut, changes in fat digestibility are improbable. However, the fat component of the HFCG supplements may have contributed to increased circulating NEFA. The average DMI of animals receiving the HFCG supplements was 23.78 kg/d, resulting in the consumption of approximately 16.6 g of HFCG per day, of which 8.3 g was composed of hydrogenated palm fat. As yields of preformed fatty acids tended to increase by approximately 30.9 g/d ($P = 0.099$), this strongly suggests the incorporation of preformed fatty acids of endogenous origin in addition to any contributions derived from the hydrogenated fat component of the supplement. Intriguingly, hindgut acidosis, and presumably hindgut barrier dysfunction, consistently lead to decreases in milk fat content, milk C18, or both (Reynolds et al., 2001; Gressley and Armentano, 2005, 2007) although the underlying mechanisms remain unknown. The fact that HFCG supplementation elicits opposite responses might be indicative of improved hindgut health, consistent with the hypothesized mode of action.

The observed production responses could be explained in part by an increase in production of VFA by hindgut microbes in response to HFCG supplementation, although we observed no difference in fecal acetate, propionate, or butyrate concentrations. This agrees with Doelman et al. (2019a) and is likely due to the rapid uptake of VFA by the gastrointestinal epithelium, limiting the value of fecal VFA content as a marker for hindgut VFA production (den Besten et al., 2013). However, it is possible to estimate the increase in VFA production in response to HFCG supplementation. Tsukahara et al. (2002) observed that gluconate was fermented to butyrate at a molar conversion ratio of 26% in vitro. Based on a 0.07% inclusion rate of HFCG in the diet and an average DMI of 23.78 kg/d,

animals in the current study received approximately 16.6 g of HFCG per day, which provided approximately 6.66 g of calcium gluconate. This is equivalent to approximately 15.47 mmol of calcium gluconate, which dissociates into 30.95 mmol of gluconate ions. Using the 26% molar conversion of Tsukahara et al. (2002), it is expected that the amount of HFCG fed would be fermented to approximately 8.05 mmol ($\sim$ 709 mg) of butyrate per day.

Similarly, it is possible to estimate the net change in butyrate incorporated into milk fat. We can assume that butyrate was used as the initial substrate for fatty acid elongation in approximately 50% of de novo fatty acids, with the other 50% being derived from acetate (Palmquist et al., 1969). The average yield of fatty acids from 4 to 14 carbons in length numerically decreased by approximately 12.30 g/d ($P = 0.431$; Figure 1) in animals supplemented with HFCG; assuming an average molecular weight of these fatty acids of 158.241 g/mol, this translates to an average decrease of 77.73 mmol, sparing approximately 38.86 mmol ($\sim$ 3.424 g/d) of butyrate. Additionally, the yield of total C16 increased by approximately 5.99 g/d ($P = 0.403$; Table 6). Assuming that 50% of total C16 was derived from de novo synthesis (Palmquist et al., 1969; Grummer, 1991; Garnsworthy et al., 2006) and an average molecular weight of 256.43 g/mol, this translates to an increase of approximately 11.68 mmol in de novo-derived C16 having been synthesized from approximately 5.84 mmol ($\sim$ 515 mg) of butyrate. Overall, we estimate that the total amount of butyrate incorporated into milk fatty acids decreased by approximately 33.02 mmol (2.909 g) per day. Although not significant, these estimations preclude the direct incorporation of calcium gluconate-derived VFA into milk to support the observed milk fat responses to HFCG supplementation.

Increased circulating NEFA in response to HFCG could be mediated through increased lipolysis, decreased re-esterification of fatty acids in adipose tissue, decreased hepatic β -oxidation, or decreased NEFA uptake and utilization by peripheral tissues (Bell, 1995; Drackley, 2001; Roche et al., 2009). However, the lack of a BW response to HFCG suggests no mobilization of body reserves, and the lack of an insulin response is suggestive of no change in lipolysis. Additionally, the trend for increased BW in the mash versus pelleted treatments suggest a net gain in body reserves in that group. However, these measures do not accurately account for potential changes in fat stores, and as BW was only evaluated during the final week of each period, we cannot comment on any potential changes that occurred while animals adapted to the HFCG supplements. Based on the increase in preformed fatty acids and specifically C18 in milk, we speculate that whole-

body NEFA utilization was reduced, which is partially supported by the concomitant decrease in blood BHB observed in HFCG-fed cows.

Although no direct measurements of gut health were performed in the current study, we hypothesize that supplementation of the prebiotic HFCG improved yields of milk fat, FCM, and ECM through its action in the hindgut in a way similar to other eubiotic interventions that have shown improved feed efficiency in growing animals (Biagi et al., 2006; Poeikhampha and Bunchasak, 2011; Michiels et al., 2020). The enhanced production of VFA in the hindgut provides a plausible mechanism for both improvements in gut health and modulation of whole-body metabolism (den Besten et al., 2013; Rios-Covian et al., 2016; Liu et al., 2018). The gastrointestinal tract is one of the most energy-demanding tissues in the body (McBride and Kelly, 1990). A disruption of intestinal barrier function can further enhance such requirements due to associated immunoactivation (Fox et al., 2005). Improving gut health can potentially alter whole-animal bioenergetics by reducing the energy/nutrient demands of the gastrointestinal tract and sparing resources toward productive purposes, such as milk production. Differences in blood parameters and milk fatty acid profile further support the hypothesis of changes in nutrient partitioning as a mode of action for the observed responses. More work is needed to accurately characterize the response to HFCG at the level of the gastrointestinal epithelium.

It is currently unclear why the yields of bioactive fatty acids (e.g., CLA, n-3, PUFA) increased in response to HFCG supplementation, as these are derived exclusively from the diet and no differences were observed in DMI. Similarly, the response in milk urea requires further investigation. The decrease in fecal isobutyrate in response to HFCG could indicate changes in protein fermentation (Allison, 1978); however, it is unclear how this would elicit a milk urea response of the magnitude observed presently. Characterizing changes in the hindgut microbial community in response to HFCG would help elucidate these findings.

In general, few differences were observed between the mash and pelleted forms of calcium gluconate supplement. Some of the effects observed (e.g., fecal isobutyrate concentration, milk fatty acid content) could reflect the partial fermentation of calcium gluconate in the rumen when fed in the pelleted form. McKnight et al. (2019) incorporated unprotected calcium gluconate into the feed of lactating dairy cattle and observed a numeric decrease in most production parameters. As no performance differences were detected between the 2 processing methods, it is likely that the majority of the calcium gluconate incorporated into the pellets was

not fermented in the rumen. More work is needed to evaluate the in situ degradability of the pelleted form.

CONCLUSIONS

Supplementing animals with a fat-embedded calcium gluconate additive tended to increase milk fat yield, and consequently yields of both FCM and ECM, while also stimulating an increase in milk urea. Milk fatty acid profiles suggest increases in the incorporation of extramammary fatty acids, both of which are supported by the observations in plasma BHB and NEFA. No major differences were observed between the 2 different processing methods used to incorporate the fat-embedded calcium gluconate supplement into the diet. More work needs to be done to characterize the effect of HFCG at the level of the gastrointestinal lumen and epithelium, as well as the responses in both milk urea and milk fatty acid profile.

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