Feeding supplemental 25-hydroxyvitamin D₃ increases serum mineral concentrations and alters mammary immunity of lactating dairy cows

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ABSTRACT

Objectives were to determine the effects of feeding supplemental 25-hydroxyvitamin D₃ [25(OH)D₃] on concentrations of vitamin D metabolites and minerals in serum, mammary immune status, and responses to intramammary bacterial infection in dairy cows. Sixty multiparous, pregnant lactating Holstein cows with somatic cell count <200,000/mL were blocked by days in milk and milk yield and randomly assigned to receive a daily top-dressed dietary supplement containing 1 or 3 mg of vitamin D₃ (1mgD or 3mgD), or 1 or 3 mg 25(OH)D₃ (1mg25D or 3mg25D) for 28 d (n = 15/treatment). Cows were kept in a freestall barn and fed a total mixed ration in individual feeding gates. Individual dry matter intake (DMI) and milk yield were recorded daily, and milk and blood samples were collected at 0, 7, 14, and 21 d relative to the start of treatment. At 21 d, cows fed 1mgD and 3mg25D received an intramammary challenge with *Streptococcus uberis*. Cows were observed for severity of mastitis, and blood and milk samples were collected every 12 h to measure inflammation. The 1mg25D and 3mg25D cows had greater serum 25(OH)D₃ concentrations at 21 d compared with 1mgD and 3mgD cows (62 ± 7, 66 ± 8, 135 ± 15, and 232 ± 26 ng/mL for 1mgD, 3mgD, 1mg25D, and 3mg25D, respectively). The 3mg25D cows had greater concentrations of Ca and P in serum at 21 d compared with other treatments (Ca = 2.38, 2.4, 2.37, and 2.48 ± 0.02 mM, 1.87, 1.88, and 2.10 ± 0.08 mM for 1mgD, 3mgD, 1mg25D, and 3mg25D, respectively). Yields of milk and milk components, DMI, body weight, and concentrations of 1,25-dihydroxyvitamin D and Mg in serum did not differ among treatments. Abundance of mRNA transcripts for interleukin-1β (*IL1B*) and inducible ni-

tric oxide synthase (*iNOS*) in milk somatic cells before *S. uberis* challenge were increased in cows fed 25(OH)D₃ compared with cows fed vitamin D₃. Furthermore, *IL1B*, *iNOS*, β-defensin 7, and β-defensin 10 in milk somatic cells increased as concentrations of 25(OH)D₃ increased in serum. Cows fed 3mg25D had less severe mastitis at 60 and 72 h after challenge with *S. uberis* compared with cows fed 1mgD. Concentrations of bacteria, somatic cells, and serum albumin in milk after challenge did not differ between treatments; however, an interaction between treatment and day was detected for lactate dehydrogenase in milk. Expression of adhesion protein CD11b on milk neutrophils after the *S. uberis* challenge was greater among 3mg25D cows compared with 1mgD cows. Transcripts of *CYP24A1* and *iNOS* in milk somatic cells during mastitis also were greater in 3mg25D cows compared with 1mgD cows. Feeding 25(OH)D₃ increased serum 25(OH)D₃ more effectively than supplemental vitamin D₃, resulting in increased serum mineral concentrations, increased expression of vitamin D-responsive genes, and altered immune responses to intramammary bacterial challenge.

Key words: vitamin D, innate immunity, dairy cow

INTRODUCTION

Nutritional interventions that improve immune defenses of dairy cows are of great value for efforts to decrease antimicrobial usage and economic burden of bacterial diseases such as mastitis and metritis in dairy cows. Recent reports have documented positive effects of vitamin D in mitigation of mastitis (Lippolis et al., 2011) and metritis (Martinez et al., 2018a). In addition to the major role of vitamin D in Ca and P homeostasis, a direct immunomodulatory role of vitamin D is documented in innate immune cells (Nelson et al., 2010b; Merriman et al., 2015) and the mammary gland (Merriman et al., 2017) of cattle.

The physiological functions of vitamin D are regulated by enzymatic activation and intracellular vitamin

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D receptors (**VDR**). Vitamin D₂ and vitamin D₃ are converted to 25-hydroxyvitamin D₂ [**25(OH)D₂**] and 25-hydroxyvitamin D₃ [**25(OH)D₃**], respectively, by 25-hydroxylases in the liver (Horst et al., 1994). Serum 25-hydroxyvitamin D [**25(OH)D**], refers to the pool of 25(OH)D₂ and 25(OH)D₃ concentrations are the most reliable indicators of vitamin D status and are between 40 to 100 ng/mL for most dairy cows fed and managed according to current practices (cows fed TMR and kept in freestall barns; Sorge et al., 2013; Nelson et al., 2016b; Holcombe et al., 2018). In contrast to 25(OH)D, the circulating concentration of the bioactive 1,25-dihydroxyvitamin D [**1,25(OH)₂D**] is tightly controlled by the activities of 1 α -hydroxylase (**CYP27B1**), an enzyme predominantly expressed in the kidneys that converts 25(OH)D to 1,25(OH)₂D, and 24-hydroxylase (**CYP24A1**), an enzyme that inactivates vitamin D metabolites in a feedback manner. Although the kidneys are the major site of 1,25(OH)₂D metabolism, many physiological functions of 1,25(OH)₂D are influenced by local metabolism of 1,25(OH)₂D in target tissues. For example, immune cell CYP27B1 converts 25(OH)D₃ to 1,25(OH)₂D₃, which then signals through the vitamin D receptor to regulate expression of genes that are critical in shaping the immune response to pathogens (Hewison, 2010). Unlike tight feedback control of the vitamin D endocrine system that contributes to Ca and P homeostasis, vitamin D signaling in immune cells is potentially more susceptible to changes in serum 25(OH)D.

Vitamin D₃ is a low-cost and effective supplement for dairy cows, but supplemental 25(OH)D₃ is an alternative to vitamin D₃ that is potentially more effective at increasing concentrations of 25(OH)D in blood of lactating dairy cows. Cows supplemented with 3 mg of 25(OH)D₃ for 21 d prepartum had serum 25(OH)D concentrations between 200 to 250 ng/mL on average, compared with 50 to 70 ng/mL for cows fed 3

mg (120,000 IU) of vitamin D₃ (Rodney et al., 2018). Cows fed 25(OH)D₃ also had improved neutrophil function, reduced risk of retained placenta, metritis, and increased milk yield compared with cows fed vitamin D₃ (Martinez et al., 2018a). Lippolis et al. (2011) also showed that intramammary 25(OH)D₃ treatment reduced severity of experimental *Streptococcus uberis* mastitis in dairy cows. We hypothesized that supplementing 25(OH)D₃ to lactating cows was more effective compared with vitamin D₃ and, consequently, supplementing 25(OH)D₃ would increase indicators of vitamin D signaling in immune cells, but not serum minerals, compared with supplementing vitamin D₃. Objectives were to evaluate the effects of 2 doses (1 mg/d or 3 mg/d) of vitamin D₃ or 25(OH)D₃ on concentrations of vitamin D metabolites, minerals, and energy metabolites in serum, indicators of immune status, and mammary immune responses to an intramammary *S. uberis* challenge among lactating dairy cows.

MATERIALS AND METHODS

Cows

The experiment was conducted at the University of Florida Dairy Research Unit. The experimental procedures and care and treatment of animals were approved by the University of Florida Institutional Animal Care and Use Committee protocol 201408677. Sixty lactating Holstein cows were enrolled in the experiment between February and May of 2016. Cows were selected for enrollment based on the following criteria: pregnant, multiparous cows with no recent mastitis, SCC < 200,000 cells/mL, average daily milk yield between 22.5 and 49.5 kg/d, and >110 DIM at enrollment (Table 1). Cows were moved to the experimental freestall barn after being selected for the experiment to acclimate to the environment and individual feeding gates (Ca-

Table 1. Descriptive statistics of lactation performance of cows used in the experiment, supplemented with 1 or 3 mg daily of either vitamin D₃ or 25-hydroxyvitamin D₃ [25(OH)D₃] in food¹

Item	Vitamin D ₃		25(OH)D ₃		SEM	P-value
	1 mg	3 mg	1 mg	3 mg		
DIM	196.6	183.8	196.5	195.1	6.0	0.38
Milk, kg/d	34.1	34.3	34.4	33.1	0.54	0.35
SCC, $\times$ 1,000/mL	90.6	64.3	54.1	45.7	3.6	<0.001
BW, kg	727.1	743.5	702.6	685.1	2.4	<0.001
Lactation	2.8	2.4	2.7	2.6	0.1	0.01

¹Cows were selected for enrollment on the basis of more than 110 DIM, milk yield between 22 and 50 kg, previous month SCC less than 200,000/mL and lactation greater than 1. Cohorts of 12 cows with similar DIM were selected for enrollment every 7 to 14 d and then blocked by milk yield within enrollment group. Data represent LSM for each treatment. Milk yield and BW represent LSM for each treatment for 2 wk before enrollment. The SCC represents LSM for each treatment from the most recent monthly DHIA test before enrollment.

lan Broadbent Feeding System, American Calan Inc., Northwood, NH).

Experimental Design and Treatments

The experiment was a randomized complete block design with a 2×2 factorial arrangement of treatments. Selection and subsequent enrollment of animals for each enrollment block was performed every 2 to 3 weeks. Cows were enrolled in groups of 12 for a total of 5 enrollment groups. Cows were blocked according to average milk yield in the 2 weeks before enrollment. All cows within enrollment group were housed together after enrollment in a freestall barn with sand-bedded stalls and capacity for 30 cows. No more than 2 enrollment groups (24 cows) were housed together at any point.

Cows were randomly assigned to an individual feeding gate on the day of enrollment. Within each block, cows were randomly assigned to receive 1 of 4 treatments. Treatments were arranged as a factorial with 2 sources of vitamin D, vitamin D₃ or 25(OH)D₃, fed at 2 amounts, 1 or 3 mg/d. Therefore, the 4 treatments consisted of 1 mg of vitamin D₃ (1mgD, 15 cows), 1 mg of 25(OH)D₃ (1mg25D, 15 cows), 3 mg of vitamin D₃ (3mgD, 15 cows), and 3 mg of 25(OH)D₃ (3mg25D, 15 cows) in addition to the base ration that provided 0.5 mg of vitamin D₃ daily. The doses were selected to reflect current industry practices for supplemental vitamin D₃ and on the basis of serum 25(OH)D, health, and production responses to 3 mg of dietary 25(OH)D₃ (Martinez et al., 2018a, b; Rodney et al., 2018). A top-dress supplement for each treatment was prepared by mixing a vitamin D₃ concentrate (DSM Nutritional Products LLC, Parsippany, NJ) or a 25(OH)D₃ concentrate (DSM Nutritional Products) with cornmeal to provide either 1 or 3 mg of each source in 100 g of the mixture. The top-dress was not mixed into the ration and was consumed readily by all cows upon delivery. Treatments were fed to all cows for 28 d. Cows in the last 2 blocks of enrollment (24 cows) received the treatments for a total of 56 d.

Cows were fed twice daily at 0700 h and again at 1430 h. The treatments were added to the morning feeding as a top-dress. The amount of TMR offered was recalculated daily to result in at least 5% but no more than 10% refusals. Refusals were weighed once daily before the morning feeding. Dry matter intake was calculated daily after weighing refusals. All cows received the same lactation diet, which was fed as a TMR (Table 2). Feed ingredients were collected twice per week. Ingredient samples were composited into the TMR and analyzed for CP, NDF, ADF, starch, and minerals at the Dairy One Forage Testing Laboratory

(Ithaca, NY). The chemical composition of the diets (mean $\pm$ SD) were calculated using the results of analyses of the TMR (Table 2).

Milk Yield and BW

Cows were milked twice daily at 1000 h and 2200 h. Milk production was recorded twice daily using Afi Milk Meters (AfiFlo milk meters, SAE Afikim, Israel). Milk and blood samples were taken on d 0, 7, 14, 21, and 28 relative to the start of treatment, into 50-mL tubes containing the preservative bronopol. Samples were submitted to the Southeast DHI laboratory in Belleview, FL, and analyzed for concentrations of fat, true protein, lactose, and SCC. Cows were weighed twice daily after each milking using a walk-through scale (AfiWeigh, SAE Afikim) as they left the parlor.

Table 2. Ingredient composition and nutrient content of postpartum diets in experiment

Item	Lactation diet
Ingredients, % DM	
Corn silage	36.1
Alfalfa hay	8.3
Brewers grains, wet	8.3
Corn, finely ground	13.9
Citrus pulp, dry	8.3
Soy hulls	5.6
Soybean meal (47% CP)	13.7
Saturated free fatty acids ¹	1.5
Mineral-vitamin protein mix ²	3.7
Mycotoxin binder ³	0.6
Nutrient content, DM basis ⁴	
NE _E , ⁵ Mcal/kg	1.67
CP, %	17.4 $\pm$ 0.2
NDF, %	30.0 $\pm$ 1.3
ADF, %	19.8 $\pm$ 2.6
Starch, %	22.6 $\pm$ 4.0
TDN, %	71.3 $\pm$ 0.6
Ca, %	0.63 $\pm$ 0.02
P, %	0.33 $\pm$ 0.02
Mg, %	0.31 $\pm$ 0.01
K, %	1.38 $\pm$ 0.13
Na, %	0.45 $\pm$ 0.03

¹Energy Booster Mag, Milk Specialties, Eden Prairie, MN.

²Mineral-vitamin-protein premix contained (% as is): 30% ProvAAL Lys and Met (Perdue Agribusiness, Salisbury, MD), 28.5% sodium bicarbonate, 13% DCAD Plus (Arm and Hammer Animal Nutrition, Trenton, NJ), 13.0% calcium carbonate, 7.0% dicalcium phosphate, 7.0% magnesium oxide, 3.5% sodium chloride, 1.2% Zinpro Availa-4 (Zinpro Corp., Eden Prairie, MN), 0.6% Sel-Plex1000 (Alltech, Nicholasville, KY), 0.22% Rumensin 90 (Elanco Animal Health, Greenfield, IN). Each kg of mineral-vitamin mix contained 149,800 IU of vitamin A, 25,700 IU of vitamin D₃, and 644 IU of vitamin E.

³NovaSilPlus, BASF, Florham Park, New Jersey.

⁴Values represent means and SD of analyzed diets. Feed ingredients were collected twice per week and analyzed at the Dairy One Forage Testing Laboratory (Ithaca, NY).

⁵Calculated using NRC (2001) according to the chemical composition of the dietary ingredients and adjusted for 20 kg of DMI.

The 2 daily BW measurements were averaged for each day.

Serum Minerals and Metabolites

Peripheral blood was sampled by puncture of the coccygeal vessels into evacuated 10-mL serum separator tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) before the beginning of treatments and again on d 7, 14, 21, and 28 relative to the start of treatment and at 24, 48, 72, and 96 h relative to the bacterial infusion for specified animals receiving the intramammary bacterial infusion. Tubes were centrifuged at $2,500 \times g$ for 15 min for serum separation within 30 min of sample collection. Serum samples were transferred into microtubes and stored frozen at -20°C .

Serum sampled on experimental d 0 and 21 was analyzed for concentrations of vitamin D₃ and D₂. Serum sampled on experimental d 21 was analyzed for concentrations of 24,25-dihydroxyvitamin D, vitamin D₃, and vitamin D₂. Both were analyzed via liquid chromatography MS at Heartland Assays (Ames, IA). Serum sampled on experimental d 0 and 21 was analyzed for concentrations of 1,25(OH)₂D via ELISA [1 α ,25(OH)₂Vitamin D ELISA, IBL-America, Minneapolis, MN] according to the manufacturer's instructions; the intra- and interassay coefficients of variation (CV) were 21.5 and 15.3%, respectively. Serum sampled on experimental d 0, 7, 14, 21, and 28 for all cows and d 42 and 56 for cows in blocks 4 and 5 of enrollment was analyzed for concentrations of 25(OH)D using ELISA [25(OH)Vitamin D ELISA, Product # VID31-K01, Eagle Biosciences, Amherst, NH]. The 25(OH)D ELISA was performed with a custom standard, as previously described (Nelson et al., 2016a), ranging from 5 to 205 ng/mL. Experimental samples were diluted 50% in the sample buffer provided with the kit so they would all be within the range of the standard curve. Known controls were similarly diluted and included in each assay plate. Intra- and interassay CV were 6.3 and 4.6%, respectively.

Serum sampled on experimental d 0, 7, 14, 21, and 28 and 24, 48, 72, and 96 h relative to the bacterial infusion was analyzed for total Ca, Mg, BHB, nonesterified fatty acid (FA), and glucose concentrations using a Randox instrument (RX Daytona, Randox Laboratories Ltd., Kearneysville, WV) with respective kits for each analyte according to the manufacturer's instructions. Respective intra- and interassay CV were 1.5 and 4.2% for Ca, 1.2 and 8.8% for Mg, 4.0 and 13.6% for BHB, 7.9 and 8.3% for FA, and 1.9 and 5.1% for glucose. Concentrations of total P were quantified in serum on experimental d 0, 7, 14, 21, and 28 using the molybdenum blue method (Quinlan and DeSesa,

1955). The intra- and interassay CV were 1.4 and 5.9% respectively.

Experimental Mastitis Challenge

The *S. uberis* (strain 0140J, gift from Dr. Geoffrey Dahl, Department of Animal Sciences, University of Florida, Gainesville) used in the challenge was first streaked onto tryptic soy agar plates (Sigma---Aldrich, St. Louis, MO) to create single colonies and incubated for 24 h at 37°C . Two colonies were removed from the plate and grown in 5 mL of Luria broth (Thermo Fisher Scientific, Waltham, MA) for 24 h, shaking at 37°C . Optical density of 1 mL of Luria broth was measured using a Bio-Rad SmartSpec 3000 (Bio-Rad Laboratories Inc., Richmond, CA). The 1-mL sample was serially diluted in sterile water to remove agar and dilute to the desired colony forming units (cfu) of 1,000/mL of sterile water. Each cow in the challenge received 3 mL of the dilution, totaling 3,000 cfu.

On experiment d 21, immediately after the morning milking, the 15 cows receiving the 1mgD treatment and the 15 cows receiving the 3mg25D treatment were challenged intramammary with 3,000 cfu of *S. uberis* suspended in 3 mL of sterile water into one quarter. The 1mgD and 3mg25D groups were selected for the challenge, as they were expected to have the greatest difference in serum 25(OH)D. The number of cfu of *S. uberis* in the milk from the noninfused control and infused infected glands was determined by culturing log dilutions of milk samples on blood agar plates for 24 h at 37°C .

Cows were monitored for rectal temperature and severity of mastitis before each milking at 0, 12, 24, 36, 48, 72, and 96 h relative to the challenge. Mastitis severity was scored on a scale of 0 to 3, with 0 being no clinical signs of mastitis; 1 as mild mastitis, including one or more of the following criterion: abnormal milk, no systematic signs, no swelling or redness of the mammary gland; 2 as moderate mastitis, including one or more of the following criterion: abnormal milk, signs of swelling or redness of the infected quarter; or 3 as severe mastitis, including one or more of the following criterion: watery milk, redness and swelling of the infected quarter, rectal temperature exceeding 39.5°C . Scoring of mastitis severity was not blinded to dietary treatments. Cows with severe mastitis, score 3, received intramammary treatment with 125 mg of ceftiofur hydrochloride (Spectramast LC, Zoetis, Florham Park, NJ) administered once daily for 5 d. If rectal temperature increased above 39.7°C , cows also received an intravenous infusion of flunixin meglumine at 2 mg/kg of BW (Banamine, Merck Animal Health, Madison, NJ).

Blood and Milk Flow Cytometry and Gene Expression

Peripheral blood leukocytes for flow cytometry were collected from peripheral blood sampled by puncture of the coccygeal vessels into evacuated 10-mL heparin tubes (Vacutainer, Becton Dickinson) on d 0, 7, 14, 21, and 28 relative to the start of treatment and at 24, 48, 72, and 96 h relative to mastitis challenge. Red blood cells were removed from 100 μ L of whole blood by hypotonic lysis. Cells were incubated 20 min on ice with allophycocyanin (APC)-conjugated anti-CD14 (Tük4 clone, Life Technologies, Carlsbad, CA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-bovine CD11b (CC126 clone, AbD Serotec, Raleigh, NC) and phycoerythrin (PE)-conjugated anti-CD62L (BAQ92A clone, Kingfisher Biotech Inc., St. Paul, MN) in 100 μ L of PBS containing 0.5% BSA (Thermo Fisher) and 2 mM EDTA (Thermo Fisher). Cells were then labeled with a fluorescent viability dye (eFuer 450, Thermo Fisher) and analyzed on the bases of (1) size and granularity, (2) viability, (3) CD14 and CD11b expression, and (4) CD62L expression, as previously described (Merriman et al., 2018), using a Sony SH800 cell sorter equipped with 405-, 488-, and 633-nm lasers (Sony Biotechnology Inc., San Jose, CA). The CD14-positive cells were considered monocytes, whereas agranular, CD14-negative cells were considered lymphocytes. Abundances of CD11b, CD14, and CD62L were determined using the median fluorescence intensity of respective fluorescent signals.

Somatic cells were collected from milk for flow cytometry and gene expression analyses. Samples were collected directly into sterile 50-mL falcon tubes before full milk withdrawal and placed directly onto ice before being transported to the laboratory. Tubes were centrifuged at $1,500 \times g$ for 20 min to separate cells, milk fat, and whey. Fat was discarded, and whey was transferred to 5-mL microtubes and frozen at -80°C until analysis. The remaining cell pellets were resuspended with 1 mL of PBS with 2 mM EDTA (Thermo Fisher). The suspension was split into 2 equal fractions: one for flow cytometry and the other for gene expression. The fraction for gene expression was transferred to a 1.5-mL tube, centrifuged at $650 \times g$ for 5 min, the PBS removed, and then resuspended in 0.5 mL of Trizol Reagent and frozen at -80°C until analysis. The somatic cells for flow cytometry were labeled and analyzed as described for peripheral blood leukocytes, except that granulocytes that were CD14-negative were considered neutrophils, and cells that were CD14-positive were considered macrophages.

For analysis of gene expression, RNA was extracted from cell samples dissolved in Trizol reagent using

Direct-zol-96 RNA Kit (ZYMO Research, Irvine, CA), according to the manufacturer's instructions, and eluted with 25 μ L of RNase-free water. The RNA was quantified via NanoDrop ND-2000 spectrophotometer (Thermo Fisher). Ribonucleic acid was reverse-transcribed to cDNA using high-capacity cDNA reverse-transcription kit (Thermo Fisher) in 20- μ L reactions that contained 10 μ L of each RNA sample and 10 μ L RNase inhibitor. Reactions were incubated at 23°C for 10 min, followed by 2 h at 37°C , and 5 min at 85°C . The cDNA samples were diluted 1:10 with DNase-free water and stored at -20°C . Quantitative PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and the CFX96 Real-Time System (Bio-Rad Laboratories Inc., Richmond, CA) with gene-specific primers (Supplemental Table S1, <https://doi.org/10.3168/jds.2019-16999>). Amplifications were performed in a 20- μ L reaction that contained 11 μ L of SYBR Select qPCR master mix (Thermo Fisher), 0.5 μ L each of 10 μ M forward and reverse primers, and 9 μ L of the 1:10 diluted cDNA sample. Reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

For determination of relative transcript abundance, the threshold cycle (Ct) for each gene in each cDNA sample was normalized to the geometric mean of the reference genes ribosomal protein S9 (*RPS9*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and β -actin (*ACTB*) using the formula $[\text{Ct}(\text{gene of interest}) - \text{Ct}(\text{geometric mean}) = \Delta\text{Ct}]$. The ΔCt values for each gene were used for statistical analysis as described below. The mean ΔCt ($\pm\text{SE}$) for each treatment was transformed using the formula $[1,000 \times 2^{-\Delta\text{Ct}}]$ to report the relative number of transcripts.

Concentrations of Inflammatory Markers in Milk

Concentrations of BSA and lactate dehydrogenase (LDH) in whey fractions of the milk samples that were collected for flow cytometry and gene expression analyses. Concentrations of BSA were measured using ELISA kits from Bethyl Laboratories (Montgomery, TX). Relative LDH abundance was measured using a colorimetric assay from Thermo Fisher Scientific. Inter- and intra-assay CV were 19 and 5% for BSA and 4 and 3% for LDH assays, respectively.

Statistical Analysis

The experiment followed a randomized complete block design with cow as the experimental unit. Lactating cows were blocked by DIM and milk yield and, within each block, assigned randomly to 1 of the 4 treatments. Continuous data were evaluated for distribu-

Table 3. Milk yield and concentration of milk components and differences between treatments¹

Item	Vitamin D ₃		25(OH)D ₃		SEM	P-value ²		
	1 mg	3 mg	1 mg	3 mg		Dose	Source	Dose × source
Yield, kg/d	32.9	33.6	32.7	32.6	0.23	0.57	0.31	0.45
DMI, kg/d	23.2	23.4	23.3	23.1	0.3	0.97	0.76	0.52
Fat, %	3.1	3.3	3.2	3.1	0.05	0.51	0.34	0.21
True protein, %	3.0	3.0	2.9	2.9	0.08	0.93	0.11	0.68
Lactose, %	4.7	4.7	4.7	4.7	0.02	0.49	0.31	0.39
SCC, log cells/mL	4.8	4.4	4.6	4.7	18	0.26	0.56	0.09

¹Cows were supplemented with 1 or 3 mg of vitamin D₃ or 25-hydroxyvitamin D₃ [25(OH)D₃] in feed. Data represent LSM of each treatment group from d 7 to 21 of dietary treatments.

²Dose = 1 mg vs. 3 mg; source = 25(OH)D₃ vs. vitamin D₃; dose × source = interaction between dose and source.

tion of the residuals and homogeneity of variance after model fitting. Data with violation of the assumptions of normality were subjected to Box-Cox transformation using the TRANSREG procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) before final statistical analyses. Data were analyzed using the MIXED procedure of SAS. Statistical models included the fixed effects of source [vitamin D₃ vs. 25(OH)D₃], dose (1 mg vs. 3 mg), day in the experiment, and interactions between dose and source, source and day, and dose and day. Random effects included block and cow nested within the interaction of dose and source of vitamin D. Measurements taken on d 0, before treatment commencement, were used as covariates for adjustment during analysis. The Kenward-Roger method was used to calculate the approximate denominator degrees of freedom to compute *F*-tests in the statistical models. The Tukey adjustment was applied to account for multiple comparisons of means when the interaction between source and dose was significant. The models for analyses of data after the challenge included the fixed effects of treatment (1mgD vs. 3mg25D), hour, and interaction between treatment and hour, and the random effect of cow nested within treatment. Associations between serum 25(OH)D and downstream vitamin D metabolites, minerals, and gene transcripts were analyzed with mixed models that included serum 25(OH)D as an independent variable with block and cow nested within interaction of source and dose as random effects. The Δ Ct values for *CYP27B1* and *VDR* transcripts were also included as explanatory variables for association of serum 25(OH)D with Δ Ct values of genes that were potential targets of VDR. Interval to diagnosis of mastitis after the challenge was analyzed by survival analysis with the LIFETEST procedure of SAS, with a model that included the effect of treatment (1mgD vs. 3mg25D). Differences with $P \leq 0.05$ were considered significant, whereas tendencies for differences were reported if $0.05 < P \leq 0.10$.

RESULTS

Of the 60 cows enrolled in the experiment, 1 cow receiving the 1mgD treatment was removed after 2 wk of treatment due to udder dermatitis that was not diagnosed before enrollment. Additionally, 1 cow receiving the 3mgD treatment was removed from the experiment 3 d after enrollment due to a bacterial infection that needed treatment. Body weight and DMI did not differ among treatments (Table 3). Milk yield and concentrations of true protein, fat, and lactose also did not differ among treatments (Table 3). A tendency ($P = 0.09$) for interaction between source and dose of vitamin D was observed because cows receiving the 3mgD treatment had reduced SCC compared with all other treatments in the first 21 d of experimental treatments (Table 3).

Concentrations of Vitamin D Metabolites in Serum

Increasing the dose of supplemental 25(OH)D₃ from 1 mg/d to 3 mg/d increased ($P < 0.001$) the concentrations of 25(OH)D in serum more effectively compared with supplemental vitamin D₃ (Table 4). Concentrations of 25(OH)D in serum were not different between 1mgD cows and 3mgD cows during the entire experiment (Table 4). In contrast, concentrations of 25(OH)D in serum of 25(OH)D₃-treated cows were increased by d 7 and continued to increase until d 28 (Table 4). For the subset of cows maintained on treatments for 56 d, concentrations of 25(OH)D remained stable from 28 d to 56 d (Table 4). Concentrations of 25(OH)D₃ in serum analyzed using mass spectrometry for a subset of cows (5 per treatment) sampled at 21 d were affected by treatment similar to total 25(OH)D measured by ELISA. Total concentrations of 25(OH)D [25(OH)D₂ and 25(OH)D₃ combined], as determined by MS, did not differ from the concentrations determined by ELISA (linear regression line of ELISA vs. MS concentrations = $1.1x - 6.5$; $P < 0.001$; $R^2 = 0.93$).

As expected, vitamin D₃ concentrations in serum were greater ($P < 0.001$) for vitamin D₃-treated cows compared with 25(OH)D₃-treated cows (Table 4). Concentrations of vitamin D₃ and 25(OH)D in serum of 1mgD and 3mgD cows were not correlated, despite greater ($P < 0.05$) concentrations of vitamin D₃ in 3mgD cows compared with 1mgD cows (Table 4 and Figure 1A). Supplementing 25(OH)D₃ did not increase serum 1,25(OH)₂D concentrations, but level of supplementation (1 mg vs. 3 mg) of either source increased ($P = 0.03$) serum 1,25(OH)₂D concentrations (Table 4). Accordingly, concentrations of 1,25(OH)₂D and 25(OH)D in serum at 21 d were not correlated (Figure 1B). Concentrations of 24,25(OH)₂D₃ in serum of 25(OH)D₃-treated cows were greater ($P < 0.001$) compared with serum 24,25(OH)₂D₃ of vitamin D₃-treated cows. Concentrations of 24,25(OH)₂D at 21 d were also correlated with 25(OH)D concentrations in serum at 21 d (Figure 1C).

Concentrations of Minerals and Energy Metabolites in Serum

Supplementing 25(OH)D₃ increased ($P = 0.006$) serum Ca concentrations compared with supplementing vitamin D₃ (Table 5). The dose of treatment (3 mg vs. 1 mg) also increased ($P = 0.03$) serum Ca concentrations (Table 5). Likewise, supplementing 25(OH)D₃ increased ($P = 0.02$) serum concentrations of P compared with supplementing vitamin D₃ (Table 5) and serum concentrations of P were greater ($P = 0.006$) for cows fed 3 mg of supplement compared with 1 mg of supplement

(Table 5). Concentrations of Mg in serum did not differ among treatments (Table 5). Serum glucose and FA concentrations were not affected by source, dose, or interaction between source and dose (Table 5); however, serum BHB tended ($P = 0.10$) to be greater for the 3-mg dose compared with the 1-mg dose. A post hoc analysis to contrast the 1mgD treatment to all other treatments also indicated that average serum BHB of 1mgD cows was less ($P = 0.009$) than the average of other treatments. The concentrations of minerals and energy metabolites in serum as a function of serum 25(OH)D also were analyzed, to determine whether any associations with serum 25(OH)D existed (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16999>). We observed that concentrations of Ca, P, and BHB in serum increased ($P < 0.01$) with serum 25(OH)D concentrations (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16999>).

Profile of Immune Cells in Blood and Milk

Peripheral blood leukocytes and milk somatic cells collected on d 7, 14, and 21 of treatment were analyzed via flow cytometry for expression of monocyte and macrophage marker CD14 and adhesion proteins CD11b and CD62L (Supplemental Table S3, <https://doi.org/10.3168/jds.2019-16999>). The percentages of lymphocytes, monocytes, and neutrophils in blood did not differ among dietary treatments. However, neutrophil CD11b expression was decreased ($P = 0.05$), and dose of treatment and monocyte CD11b tended to be decreased by source and dose of treatment (Supplemen-

Table 4. Effects of treatments on serum concentrations of vitamin D metabolites¹

Metabolite ²	Day	Vitamin D ₃		25(OH)D ₃		SEM	P-value ³		
		1 mg	3 mg	1 mg	3 mg		Dose	Source	Dose × source
Vitamin D ₃ , ng/mL	21	6.7 ^b	15.4 ^c	1.3 ^a	1.4 ^a	1.1	<0.01	<0.01	<0.01
25(OH)D, ng/mL*	7	50 ^a	55 ^{ab}	77 ^b	125 ^c	8	<0.01	<0.01	<0.01
	14	59 ^a	62 ^a	107 ^b	190 ^c				
	21	63 ^a	68 ^a	140 ^b	230 ^c				
	28	60 ^a	77 ^a	160 ^b	272 ^c				
	56 ⁴	57 ^a	77 ^a	180 ^b	278 ^c				
25(OH)D ₂ , ng/mL	21	9.6	6.9	11.3	9.8	0.8	0.01	<0.01	0.44
25(OH)D ₃ , ng/mL	21	56 ^a	57 ^a	133 ^b	254 ^c	10	<0.01	<0.01	<0.01
1,25(OH)D, pg/mL	21	15	20	17	22	2	0.03	0.45	0.94
24,25(OH) ₂ D ₂ , ng/mL	21	0.7	0.6	0.9	1.1	0.1	0.87	<0.01	0.25
24,25(OH) ₂ D ₃ , ng/mL	21	5.4 ^a	3.9 ^a	10.8 ^a	29.1 ^b	2.5	0.04	<0.01	<0.01

^{a-c}Means with different letter indicates values are different, $P < 0.05$.

¹Cows were supplemented with 1 or 3 mg of vitamin D₃ or 25-hydroxyvitamin D₃ [25(OH)D₃] in feed. Data represent LSM for cows from last 2 enrollment blocks (n = 6 per treatment) that were fed treatments for 56 d.

²Vitamin D₃, 25-hydroxyvitamin D₂ [25(OH)D₂], 25(OH)D₃, 24,25-dihydroxyvitamin D₂ [24,25(OH)₂D₂], and 24,25(OH)₂D₃ metabolites in serum were measured via liquid chromatography and tandem MS in subset of cows (n = 5 per treatment). The 25(OH)D and 1,25(OH)₂D are measures of combined concentrations of D₂ and D₃ metabolites in serum, as determined using ELISA.

³Dose = 1 mg vs. 3 mg; source = 25(OH)D₃ vs. vitamin D₃; dose × source = interaction between dose and source.

*Interaction among source, dose, and day was significant, $P < 0.05$.

Figure 1. Concentrations of vitamin D₃ (A), 1,25-dihydroxyvitamin D [1,25(OH)₂D] (B), and 24,25-dihydroxyvitamin D [24,25(OH)₂D] (C) in serum vs. concentrations of 25-hydroxyvitamin D [25(OH)D] in serum at 21 d after feeding 1 or 3 mg of vitamin D₃ (1mgD or 3mgD) or 1 or 3 mg of 25-hydroxyvitamin D₃ [25(OH)D₃; 1mg25D or 3mg25D] per day. The 24,25(OH)₂D and vitamin D₃ metabolites were measured in a subset (n = 5) of cows in each group. In panels B and C, lines represent linear regression between 1,25(OH)₂D ($y = 0.01x + 17.7$; $P = 0.45$) and 24,25(OH)₂D₃ ($y = 0.12x - 3.1$; $P < 0.001$).

tal Table S3, <https://doi.org/10.3168/jds.2019-16999>). Dietary treatments did not affect the percentages of macrophages or neutrophils in milk, nor did treatment affect CD14 or CD62L expression on milk somatic cells (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-16999>).

Gene Expression in Milk Somatic Cells Before Mastitis Challenge

Treatments did not affect the expression of *CYP27B1*, the gene coding the enzyme that converts 25(OH)D to 1,25(OH)₂D, in milk somatic cells (Figure 2). Expression of *VDR*, however, tended to be increased ($P = 0.1$) by dose of treatment, as cows fed 3-mg doses showed greater expression of *VDR* than did cows fed the 1-mg dose. Expression of *VDR* in milk somatic cells did not differ between sources of vitamin D treatment.

Because activity of 25(OH)D₃ in immune cells is reported to depend on activity of *CYP27B1* and *VDR*, the levels of *CYP27B1* and *VDR* in milk somatic cells were used as explanatory variables in the model to analyze effects of dietary treatment on all other genes. Treatments did not affect the expression of *CYP24A1*, which is induced by 1,25(OH)₂D and encodes for the enzyme that catabolizes 25(OH)D and 1,25(OH)₂D metabolites. Likewise, treatments did not affect expression of β -defensins (*DEFB3*, *DEFB4*, *DEFB7*, and *DEFB10*) or lingual antimicrobial peptide (*LAP*; Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-16999>). The chemokines *CCL2* and *CCL5* did not differ between vitamin D₃ and 25(OH)D₃ treatments, but *CCL2* was greater ($P = 0.04$) in milk somatic cells of cows fed 1 mg of vitamin D₃ or 25(OH)D₃ compared with cows fed 3 mg of either metabolite (Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-16999>). Expression of *IL1B* in milk somatic cells was 1.7-fold greater ($P = 0.01$) in cows fed 25(OH)D₃ compared with cows fed vitamin D₃ (Figure 2). Finally, expression of *iNOS* in milk somatic cells was 1.4-fold greater ($P = 0.01$) in cows fed 25(OH)D₃ compared with vitamin D₃, but *iNOS* was less abundant in cows fed 3 mg of vitamin D₃ or 25(OH)D₃ compared with cows fed 1 mg of either metabolite (Figure 2). Expression of *iNOS* also was greater ($P = 0.02$) for cows fed 25(OH)D₃ compared with those fed vitamin D₃, if *CYP27B1* and *VDR* were excluded as variables in the model.

The association of serum 25(OH)D₃ and gene expression in milk somatic cells was analyzed via linear regression (Table 6). Concentrations of 25(OH)D₃ in serum were not associated with expression of *CYP27B1* or *VDR*. The analyses for the remaining genes included *CYP27B1* and *VDR* as covariates. Expression of *CYP24A1* in milk somatic cells increased ($P = 0.02$) as

Table 5. Effects of treatments on serum concentrations of minerals and energy metabolites¹

Analyte	Vitamin D ₃		25(OH)D ₃		SEM	P-value ²		
	1 mg	3 mg	1 mg	3 mg		Dose	Source	Dose × source
Ca, mM	2.36	2.37	2.38	2.44	0.02	0.03	0.01	0.17
P, mM	1.76	1.90	1.87	2.14	0.07	<0.01	0.02	0.39
Mg, mM	0.95	0.96	0.94	0.97	0.02	0.24	0.85	0.50
BHB, mM*	0.53	0.61	0.60	0.60	0.02	0.10	0.16	0.12
Glucose, mM	3.82	3.75	3.67	3.77	0.06	0.82	0.32	0.14
FA, μM	67	61	59	61	3	0.53	0.27	0.26

¹Cows were supplemented with 1 or 3 mg of vitamin D₃ or 25-hydroxyvitamin D₃ [25(OH)D₃] in feed. Data represent LSM for samples collected at 7, 14, and 21 d. Concentrations at 0 d were used as a covariate in the model.

²Dose = 1 mg vs. 3 mg; source = 25(OH)D₃ vs. vitamin D₃; dose × source = interaction between dose and source. The effects of day and interaction among source, dose, and day for each analyte were not significant.

*Contrast of 1 mg of vitamin D₃ vs. other treatments is significant, $P = 0.009$.

serum 25(OH)D₃ increased. The β -defensins *DEFB3*, *DEFB4*, and *LAP* were not affected by serum 25(OH)D₃, whereas *DEFB7* and *DEFB10* increased ($P < 0.05$) with serum 25(OH)D. The chemokines *CCL2* and *CCL5* were not affected by serum 25(OH)D; however, *IL1B* and *iNOS* increased with serum 25(OH)D (Table 6).

Effect of Dietary Treatment on Mastitis Challenge

Incidence of clinical mastitis did not differ between 1mgD and 3mg25D cows. For both treatments, 10 cows developed clinical signs of mastitis after the bacterial challenge (1mgD $n = 10$ out of 14; 3mg25D $n = 10$ out of 15). Treatment did not affect the time to onset of clinical symptoms (Figure 3). Two cows in the 1mgD group developed severe mastitis by 72 h and received antibiotic and anti-inflammatory treatments at 72 h, but time to antibiotic treatment did not differ between treatments (Figure 3). Milk yield and DMI declined (effect of day, $P < 0.001$) after the challenge in both treatments, but milk yield and DMI did not differ between treatments from d 21 to 28 (Figure 3).

Mastitis severity was greater ($P < 0.05$) in 1mgD cows compared with 3mg25D cows at 60 and 72 h (Figure 4A). At 96 h there were no differences in mastitis incidence or severity between the 2 treatments. Rectal temperatures increased ($P < 0.001$) with time, but the 1mgD and 3mg25D cows (39.2 vs. $39.0 \pm 0.08^\circ\text{C}$) did not differ (Figure 4B). The SCC in milk increased ($P < 0.05$) over time but did not differ between treatments (Figure 4C); however, data were missing for 12 cows (6 per treatment) because samples were damaged or spoiled during shipping to the laboratory that performed milk SCC assays. Bacterial counts in milk increased ($P < 0.001$) over time to a maximum of approximately 1×10^5 cfu/mL at 60 h, but they were not different between treatments (Figure 4D). Concentrations of BSA in milk whey increased ($P < 0.05$) after the challenge (Figure

4E) but were not affected by dietary treatment. An interaction between treatment and time ($P = 0.03$) was detected for LDH activity in milk after the challenge, such that the pattern of increase in LDH over time after challenge differed between the 1mgD and 3mg25D treatments (Figure 4F).

The percentage of macrophages in milk after the challenge was greater than before challenge but decreased over time during the challenge (Figure 5A). The percentage of neutrophils in milk increased ($P < 0.001$) over time from 46% before the challenge to 65% at 72 h (Figure 5B). Expression of adhesion protein CD11b on macrophages and neutrophils decreased over time after the challenge, and expression of CD11b was greater on macrophages ($P = 0.09$; Figure 5C) and neutrophils ($P = 0.05$; Figure 5D) of 3mg25D cows compared with 1mgD cows. Expression of adhesion protein CD62L on macrophages increased over time after the challenge ($P = 0.01$) but did not differ between treatments (Figure 5E). In contrast, a tendency for interaction between treatment and time ($P = 0.07$) was detected for neutrophil CD62L, such that a decrease in neutrophils CD62L was observed in 1mgD cows from 24 h to 48 h, whereas neutrophil CD62L in 3mg25D cows decreased from 48 to 72 h (Figure 5F). Treatments did not affect concentrations of lymphocytes, monocytes, or neutrophils in blood after the challenge, but a tendency for interaction between treatment and time ($P = 0.08$) was detected for monocyte CD14 expression, such that monocyte CD14 increased slightly for 1mgD cows after challenge but slightly decreased for 3mg25D cows (Supplemental Figure S2, <https://doi.org/10.3168/jds.2019-16999>).

Expression of *CYP27B1* increased ($P < 0.001$) nearly 8-fold at 48 h after challenge, relative to prechallenge values (Figure 6). Likewise, *VDR* expression increased ($P = 0.03$) in milk somatic cells after the bacterial challenge. Neither *CYP27B1* nor *VDR* were affected by dietary treatment. Similar to analysis of gene expres-

sion in milk somatic cells before challenge, *CYP27B1* and *VDR* in milk somatic cells were used as covariates in the model to analyze effects of dietary treatment on all other genes. Expression of *CYP24A1* increased ($P < 0.001$) over time and was 2-fold greater ($P = 0.02$) in milk somatic cells of 3mg25D cows compared with 1mgD cows in the postchallenge period. Likewise, *iNOS* expression increased ($P < 0.001$) over time and was 1.6-fold greater ($P = 0.02$) from 36 to 72 h after bacterial challenge. Transcripts for β -defensins *DEFB7* and *DEFB10* were not affected by treatment. In contrast, an interaction between treatment and time was detected ($P < 0.05$) for *DEFB3*, *DEFB4*, and *LAP* in the first 36 h after challenge, such that expression increased sharply at 36 h in 1mgD cows but remained constant or slightly decreased in 3mg25D cows from 12

to 36 h after the challenge. The chemokines *CCL2* and *CCL5* and cytokine *IL1B* changed over time ($P < 0.05$) but were not affected by dietary treatments.

DISCUSSION

Mastitis and metritis account for a large proportion of therapeutic antimicrobial usage in lactating dairy cows (Oliver et al., 2011). A better understanding of natural defenses against bacterial infections is needed, to decrease antimicrobial usage and lost productive output of dairy cows. Evidence has accumulated in the last decade that vitamin D signaling contributes to activation of immune defenses of the mammary gland in addition to its well-known role in mineral and skeletal homeostasis (Nelson et al., 2010a; Lippolis et al.,

Figure 2. Effects of source and dose of supplemental vitamin D on gene expression in milk cells before mastitis challenge. Cows were fed 1 or 3 mg of vitamin D₃ (1mgD or 3mgD) or 1 or 3 mg of 25-hydroxyvitamin D₃ (1mg25D or 3mg25D); $n = 15$ per treatment. Abundance of transcripts (Rel. mRNA Abundance) for cytochrome P450 family B1 (*CYP27B1*), cytochrome P450 family 24A1 (*CYP24A1*), vitamin D receptor (*VDR*), β -defensins (*DEFB7*), interleukin 1 β (*IL1B*), and inducible nitric oxide synthase (*iNOS*) were measured by quantitative PCR and reported relative to geometric mean of β -actin (*ACTB*), *GAPDH*, and ribosomal protein S9 (*RPS9*). Data represent the relative transcript abundance from transformation ($2^{-\Delta Ct}$) of ΔCt LSM $\pm$ SE of samples from d 7, 14, and 21 of treatments (Ct = threshold cycle). Effects of source [vitamin D₃ vs. 25(OH)D₃], dose (1 mg vs. 3 mg), and interaction between source and dose are indicated on each plot.

Table 6. Associations between serum 25-hydroxyvitamin D [25(OH)D] and gene expression in milk somatic cells¹

Gene ²	25(OH)D			CYP27B1			VDR		
	Coeff. ³	SE	P-value	Coeff.	SE	P-value	Coeff.	SE	P-value
<i>CYP27B1</i>	−0.8	1.8	0.67	—	—	—	—	—	—
<i>VDR</i>	0.5	0.1	0.63	—	—	—	—	—	—
<i>CYP24A1</i>	3.6	1.6	0.03	0.04	0.07	0.63	0.65	0.17	<0.01
<i>DEFB3</i>	0.6	3.5	0.87	0.22	0.15	0.14	−0.07	0.30	0.83
<i>DEFB4</i>	2.1	1.5	0.19	0.21	0.07	<0.01	0.11	0.13	0.41
<i>DEFB7</i>	3.2	1.5	0.04	0.29	0.06	<0.01	0.37	0.12	<0.01
<i>DEFB10</i>	4.1	1.9	0.03	0.42	0.08	<0.01	0.37	0.17	0.03
<i>LAP</i>	0.3	1.5	0.82	0.21	0.06	<0.01	0.24	0.13	0.07
<i>CCL2</i>	3.2	2.0	0.13	0.16	0.09	0.10	−0.09	0.19	0.65
<i>CCL5</i>	1.4	2.1	0.51	−0.08	0.10	0.43	0.07	0.19	0.71
<i>IL1B</i>	7.7	2.2	<0.01	0.21	0.11	0.05	0.95	0.21	<0.01
<i>iNOS</i>	4.0	1.6	0.01	0.54	0.07	<0.01	0.35	0.13	<0.01

¹Gene expression in milk somatic cells and concentrations of 25(OH)D in serum were measured at d 0, 7, 14, and 21 of the experiment. The associations between serum 25(OH)D and gene expression as ΔCt [$\text{Ct}(\text{gene of interest}) - \text{Ct}(\text{control genes}) = \Delta\text{Ct}$, where Ct = quantitative PCR threshold cycle] were analyzed with a mixed model that included fixed effect of serum 25(OH)D and random effect of cow nested within block and treatment. The ΔCt of *CYP27B1* and *VDR* were also included in the model, as effects of 25(OH)D in immune cells depend on immune cell *CYP27B1* and *VDR* abundance.

²Gene abbreviations: *CYP27B1* = cytochrome P450 family 27B1, *VDR* = vitamin D receptor, *CYP24A1* = cytochrome P450 family 24A1, *DEFB* = β -defensin, *LAP* = lingual antimicrobial peptide, *CCL* = chemokine (CC) motif ligand, *IL1B* = interleukin 1 β , *iNOS* = inducible nitric oxide synthase.

³The coefficient for effect of serum 25(OH)D was multiplied by -1×10^3 for reporting purposes because the ΔCt decreases as transcript abundance increases. Using the transformation of $2^{-\Delta\text{Ct}}$, a coefficient of 4 equates to a 3% increase in transcript abundance for each 10 ng/mL increment of serum 25(OH)D.

2011; Merriman et al., 2017). Thus, investigation of the effects of dietary vitamin D on mammary immunity and mastitis resistance holds promise for decreasing the negative effects of mastitis.

Mammary Immunity and Response to Bacterial Challenge

A key objective of the experiment was to investigate the effects of increased concentrations of 25(OH)D on mammary gland immunity and mammary immune response to bacterial challenge. Recent studies have provided strong evidence that vitamin D, acting through an intracrine signaling pathway of innate immune cells, activates innate defenses of the mammary gland (Merriman et al., 2017, 2018). Here, we found that supplementing 25(OH)D₃ lessened the severity of mastitis but increased milk leukocyte CD11b and CD62L protein expression and *iNOS* gene expression in response to *S. uberis* challenge. Before the challenge, we found that supplementing 25(OH)D₃ increased milk leukocyte expression of *IL1B* and *iNOS* and that *DEFB7*, *DEFB10*, *IL1B*, and *iNOS* increased with serum 25(OH)D. The results of the present experiment give further evidence in support of the role of vitamin D signaling in mammary immunity, which we propose contributes to protection of the mammary gland from mastitis.

Unexpectedly, we also observed that the 3 mg/d dose of vitamin D₃ or 25(OH)D₃, compared with 1 mg/d,

decreased peripheral blood monocyte and neutrophil CD11b expression before the mastitis challenge. The CD11b partners with CD18 to form the Mac-1 complex that is key to leukocyte extravagation by facilitating leukocyte adhesion to endothelial and epithelial surfaces (Smits et al., 2000). Increased peripheral blood leukocyte CD11b is a diagnostic marker of inflammation, although before the challenge all cows were apparently healthy. In contrast, after the intramammary *S. uberis* challenge, CD11b was increased on macrophages and neutrophils in milk of the challenged quarters of 3mg25D cows compared with 1mgD cows. The percentage of CD11b-positive cells was also increased in 3mg25D cows compared with 1mgD cows. Another key adhesion molecule, CD62L, also tended to be increased on macrophages and neutrophils from milk of 3mg25D cows compared with 1mgD cows. Similar results for CD11b were reported by Merriman et al. (2018) in response to intramammary 25(OH)D₃ and LPS challenge. These findings potentially suggest that increased 25(OH)D₃ improves leukocyte recruitment to the mammary gland upon bacterial challenge. On the other hand, in the absence of bacterial challenge, increased supplies of vitamin D keep systemic inflammation in check, which is manifested by decreased CD11b on peripheral blood leukocytes.

The functional outcomes from feeding 25(OH)D₃ in regard to mastitis are far from certain, but we speculate that increasing the supply of 25(OH)D₃ for mam-

mary immune cells boosts protection of the mammary gland against mastitis. Cows fed the 3mg25D treatment had delayed onset of mastitis symptoms compared with 1mgD cows, as indicated by severity scores. Similarly, Lippolis et al. (2011) reported that intramammary 25(OH)D₃ treatment decreased bacterial and somatic cell counts in milk after intramammary *S. uberis* challenge. In both experiments, 25(OH)D₃ treatment did not prevent mastitis, and antibiotic treatment was needed to clear the intramammary infection. Regardless, supplementing 25(OH)D₃ either by diet or intramammary changes the dynamics for onset of mastitis from *S. uberis* challenge. Caution must be used when interpreting the clinical and cellular responses to the challenge, because the intramammary challenge model

does not represent a naturally occurring infection. In this challenge model, 3,000 cfu of *S. uberis* were infused into the gland, which bypasses and overwhelms the natural defenses of the udder. Normally, opportunistic pathogens, such as *S. uberis*, that are able to evade the physical defenses of the udder establish infection if the resident defenses of the udder are not able to eliminate the invading bacterium. Risk for intramammary infection, consequently, is increased for cows with suppressed immune systems. The cows used in this experiment were in mid- to late lactation and at low risk for mastitis compared with early-lactation cows. Therefore, the model does not depict natural infection or status of the cows that are most susceptible to infection.

Figure 3. Clinical outcomes of intramammary *Streptococcus uberis* challenge. Cows fed the 1-mg vitamin D₃ (1mg VitD) and 3-mg 25-hydroxyvitamin D₃ [25(OH)D₃; 3mg 25D] treatments received an intramammary challenge with *S. uberis* at 21 d. (A) Survival curve of percentages of cows without mastitis (no difference between treatments, $P = 0.40$); (B) survival curve of percentages of cows without antibiotic treatment (no difference between treatments, $P = 0.67$) over time. Final incidence of mastitis was 10 out of 15 for each treatment. Antibiotic treatment was initiated upon detection of symptoms of severe mastitis or after 48 h of moderate mastitis. Data represent the LSM $\pm$ SE of milk yield (C) and feed intake (D) during the challenge period. Effects of treatment (Trt) and interaction between treatment and time (Trt $\times$ Time) are indicated on each plot.

Figure 4. Effects of treatment on indicators of mastitis severity. Cows fed 1-mg vitamin D₃ (1mgD) and 3-mg 25-hydroxyvitamin D₃ [25(OH) D₃; 3mg25D] treatments were challenged with *Streptococcus uberis* at d 21 of treatment. Data represent LSM $\pm$ SE of mastitis severity (A) and rectal temperatures (B), and concentrations of somatic cells (C), bacteria (D), BSA (E), and absorbance at 450 nm (ABS₄₅₀) of lactate dehydrogenase (LDH; F) in milk during the challenge period. Mastitis severity was scored on a scale of 0 to 3, where 0 = no mastitis, 1 = mild mastitis (small flakes in milk), 2 = moderate mastitis (chunks in milk with minor swelling of quarter), 3 = severe mastitis (large chunks, watery milk, swelling, and fever). Effects of treatment (Trt) and interaction between treatment and time (Trt $\times$ Time) are indicated on each plot. *Indicates significant difference between treatments ($P < 0.05$) for the indicated time.

If vitamin D does indeed contribute to protection from mastitis, the mechanisms by which it does so remain to be elucidated. The limited number of immune parameters we measured in the mammary gland does not come close to representing the complexity of host-defense mechanisms of the mammary gland (Aitken et al., 2011). The genes evaluated in this experiment were previously reported to have some association with vitamin D signaling in the immune system (Merriman et al., 2015). We are fairly confident the effects of 25(OH)D₃ occur via intracellular vitamin D signaling in macrophages and neutrophils in the mammary gland. Macrophages and neutrophils in the mammary gland express the CYP27B1 enzyme that converts 25(OH)D to 1,25(OH)₂D (Merriman et al., 2018), and 1,25(OH)₂D₃ acts in macrophages, mammary epithelial cells, or neutrophils to increase genes such as *iNOS*

and *DEFB7* (Merriman et al., 2015). As previously reported, *CYP27B1* and *VDR* in milk somatic cells were increased during mastitis in this experiment. Even before mastitis, the genes affected by 25(OH)D₃ (*DEFB7*, *iNOS*, and *IL1B*) also were dependent on *CYP27B1* and *VDR* expression in milk somatic cells. Thus, the evidence is fairly strong that CYP27B1 and VDR activity in immune cells contributes to the effects of 25(OH)D₃, but the question remains as to how vitamin D signaling in the immune system protects against bacterial infection. Increased bactericidal activity by nitric oxide and β -defensin arguably has a role in mediating effects of 25(OH)D₃ (Selsted et al., 1993; García-Barragán et al., 2018). Increased neutrophil recruitment from elevated CD11b and CD62L is also likely to mediate effects of 25(OH)D₃ (Smits et al., 2000). Vitamin D signaling also is reported to limit inflammatory signals and improve

Figure 5. Effects of treatment on leukocytes in milk of quarters challenged with intramammary *Streptococcus uberis*. Cows fed 1-mg vitamin D₃ (1mgD) and 3-mg 25-hydroxyvitamin D₃ [25(OH)D₃; 3mg25D] treatments were challenged with *S. uberis* at d 21 of dietary treatments. Cells were analyzed by flow cytometry first on the basis of size, granularity, and viability and then on the basis of CD14 expression to identify macrophages (CD14-positive) and neutrophils (CD14-negative and granular). Expression of adhesion proteins CD11b and CD62L on cells were indicated by median fluorescence intensity (MFI) of CD11b-specific and CD62L-specific antibodies. Data represent LSM $\pm$ SE of concentrations of macrophages (A) and neutrophils (D) in milk, expression of CD11b on macrophages (B) and neutrophils (E), and expression of CD62L on macrophages (C) and neutrophils (F) during the mastitis challenge. The effect of time was significant ($P < 0.05$) for each parameter. Effects of treatment (Trt) and interaction between treatment and time (Trt $\times$ Time) are indicated on each plot.

Figure 6. Gene expression in milk cells during mastitis challenge. Cows fed the 1-mg vitamin D₃ (1mgD) and 3-mg 25-hydroxyvitamin D₃ [25(OH)D₃; 3mg25D] treatments were challenged with *Streptococcus uberis* at d 21 of treatments. Milk somatic cells were collected from challenged quarters at 12, 24, 36, 48, 72, and 96 h postchallenge for analysis of gene expression. Abundance of transcripts for cytochrome P450 family B1 (*CYP27B1*), cytochrome P450 family 24A1 (*CYP24A1*), vitamin D receptor (*VDR*), β -defensins (*DEFB3*, *DEFB4*, *DEFB7*, and *DEFB10*), chemokine (CC) motif ligands (*CCL2* and *CCL5*), interleukin 1 β (*IL1B*), lingual antimicrobial peptide (*LAP*), and inducible nitric oxide synthase (*iNOS*) were measured by quantitative PCR and reported relative to geometric mean of β -actin (β -Actin), *GAPDH*, and ribosomal protein S9 (*RPS9*). Data represent relative transcript abundance (Rel. mRNA Abundance) from the transformation ($2^{-\Delta Ct}$) of ΔCt LSM $\pm$ SE (Ct = threshold cycle). Effects of treatment (Trt) and interaction between treatment and time (Trt $\times$ Time) during the entire challenge period (12 to 96 h) are indicated on each plot. *Indicates effect of treatment ($P < 0.05$) for the indicated period of time; #indicates interaction between treatment and time ($P < 0.05$) for the indicated period of time.

epithelial barrier integrity (Zhao et al., 2012; Hoe et al., 2016). However, experiments are still needed to elucidate the effects of vitamin D signaling on key functions of mammary immunity, such as bactericidal activity, neutrophil recruitment, and resolution of inflammation.

Vitamin D Nutrition and Physiology

Our data has revealed some key points in regard to vitamin D metabolism that have implications for vitamin D nutrition of dairy cows. We found that dietary 25-hydroxyvitamin D₃ was much more effective in increasing the concentrations of 25(OH)D in serum compared with vitamin D₃. In fact, serum 25(OH)D concentrations did not differ with an increased dose of vitamin D₃ from 1 to 3 mg/d (total of 1.5 mg and 3.5 mg of vitamin D₃/d), suggesting that simply feeding more than 1.5 mg/d of vitamin D₃ is not effective at increasing serum 25(OH)D for mid- to late-lactation dairy cows. Concentrations of 25(OH)D in the blood of dairy cows fed diets supplemented with vitamin D₃ typically range from 40 to 100 ng/mL (Nelson et al., 2016b). The lack of significant increase in serum 25(OH)D of 3mgD cows compared with 1mgD cows challenges the assumption that serum 25(OH)D of cows will continue to rise with supplemental vitamin D₃. However, the doses of supplemental vitamin D₃ we used were well above requirements for normal homeostasis, and a dose response to supplemental vitamin D₃ will occur at lower doses of supplemental vitamin D₃ (McDermott et al., 1985).

Vitamin D₃ is converted to 25(OH)D₃ by 25-hydroxylases in the liver (Casas et al., 2013). Generally, it has been thought that conversion of vitamin D₃ to 25(OH)D₃ is, for the most part, unregulated (Hughes et al., 1977). Based on the concentrations of vitamin D₃ in the 1mgD and 3mgD treatments, we hypothesize that 25-hydroxylase activity in the liver is regulated in the dairy cow to maintain serum 25(OH)D concentrations somewhere between 40 to 100 ng/mL. This hypothesis is supported by studies of 25-hydroxylase activity of rats, in which activity is decreased by supplementation of vitamin D₃ (Bhattacharyya and DeLuca, 1973; Bolt et al., 1988). These results suggest that 25-hydroxylase acts similarly to many other physiological feedback mechanisms, and enzyme activity is reduced when substrate is overwhelmingly available. Practically, this has importance for vitamin D nutrition, in that supplementation of cows with high doses of vitamin D (i.e., >1.5 mg vitamin D₃/d) potentially has no benefit for health and production of cows. On the other hand, a benefit of increasing supplemental vitamin D₃ from present recommendations of 0.5 mg/d (20,000 IU; NRC, 2001)

to 1 or 1.5 mg/d cannot be ruled out on the basis of this experiment.

Our findings also provide insight on safety concerns of supraphysiological serum concentrations of 25(OH)D (i.e., 100 to 300 ng/mL) in lactating dairy cows. Toxicity caused by vitamin D results in calcification of tissues (Littledike and Horst, 1982). Calcification resulting from vitamin D toxicity is thought to result in unregulated VDR activity and, consequently, increased absorption and retention of Ca (Jones, 2008). Littledike and Horst (1982) suggested that vitamin D toxicity in dairy cows begins to occur as 25(OH)D concentrations exceed 200 ng/mL. The limit of 200 ng/mL proposed by Littledike and Horst (1982), however, was based on experiments with massive bolus doses of vitamin D₃. The authors observed that cows with serum 25(OH)D concentrations greater than 200 ng/mL were more likely to develop symptoms of vitamin D toxicity. Here, although serum Ca concentrations were slightly elevated, we did not observe indications of hypercalcemia, despite serum 25(OH)D over 200 ng/mL. Serum 25(OH)D concentrations exceeded 300 ng/mL in cows fed the 3mg25D treatment, which resulted in a slight increase (0.15 mM) in serum Ca, but Ca concentrations of those cows remained below the threshold of 2.7 mM that is considered hypercalcemic (Littledike and Horst, 1982). Celi et al. (2018) did not observe any adverse histological or pathological effects from feeding 25(OH)D₃ up to 8.5 µg/kg of BW to dairy calves for 90 d. Martinez et al. (2018a) and Weiss et al. (2015) also did not observe signs of hypercalcemia with serum 25(OH)D > 200 ng/mL during the prepartum period. Thus, for short-term applications, such as the periparturient period, feeding 25(OH)D₃ to achieve supraphysiological concentrations of 25(OH)D in serum does not seem to be cause for concern of vitamin D toxicity.

In addition to expected correlations between serum 25(OH)D and serum minerals, we observed that serum BHB increased with serum 25(OH)D. The cause for the relationship between vitamin D and BHB is unknown. No changes were detected in glucose or FA concentrations in serum here, so changes in BHB are inconsequential to FA and glucose in serum. Martinez et al. (2018a) reported a tendency for increased postpartum BHB for cows fed 3 mg 25(OH)D prepartum compared with cows fed 3 mg vitamin D₃. Vieira-Neto et al. (2017) also reported that a one-time subcutaneous injection of 300 µg of 1,25(OH)₂D₃ to postpartum cows increased serum BHB compared with placebo injection. Postpartum oral administration of a single Ca bolus increased serum BHB in cows at low risk for metritis (Martinez et al., 2016); however, the increased serum Ca from the oral Ca bolus was transient, and the elevated BHB

concentrations of Ca-treated cows were present when serum Ca was lesser than placebo cows. A relationship between supplemental vitamin D and serum BHB without changes in serum Ca was also observed in weaned Holstein bull calves, instead indicating that the effect of 25(OH)D₃ on serum BHB may be independent of Ca (Blakely et al., 2019). Another potential link to explore between vitamin D and BHB is repression of pyruvate carboxylase (PC) by vitamin D. The PC is under negative control of vitamin D response elements in the PC gene promoter, and 1,25(OH)₂D₃ suppresses PC activity in human mammary epithelial and adipose cell lines (Wilmanski et al., 2017; Larrick et al., 2018). A reduction in PC activity would hinder entry of pyruvate into the TCA cycle, instead shuttling it toward acetyl-CoA and potentially greater BHB synthesis. Determining a mechanism linking serum 25(OH)D to BHB has practical significance for dairy cows, as ketosis is a common metabolic condition in postpartum cows.

CONCLUSIONS

Feeding 25(OH)D₃ was more effective than vitamin D₃ at increasing serum 25(OH)D in lactating cows. In fact, our data suggest there may be little to no benefit of supplementing vitamin D₃ above 1.5 mg/d (60,000 IU/d) with respect to increasing serum 25(OH)D. The greater serum 25(OH)D that resulted from feeding 25(OH)D₃ increased serum Ca and P, which may have implications for improving bone mineralization of lactating cows but also warrants concerns for toxicity from long-term exposure to supplemental 25(OH)D₃. The effects of feeding 25(OH)D₃ on mammary immunity and immune responses to intramammary *S. uberis* challenge support a role for vitamin D signaling in protection of the mammary gland from mastitis. Future experiments need to elucidate the effects of 25(OH)D₃ on immunoprotective functions of macrophages, mammary epithelial cells, and neutrophils in the mammary gland. Our data also warrant future experiments to test the effects of feeding 25(OH)D₃ on incidence and resolution of bacterial diseases on a larger scale.

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