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Influence of Deliverable Form of Dietary Vitamin D_3 on the Immune Response in Late-Lactating Dairy Goats

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Abstract: Mastitis-causing bacteria can establish persistent infections in the mammary glands of commercially important dairy animals despite the presence of strong specific humoral and cellular immune mechanisms. We investigated the effect of vitamin D_3 in the diet at a set level, but in two different forms (i.e., unencapsulated and encapsulated by complex coacervation with sulfur-saturated bovine lactoferrin-alginate using microbial transglutaminase-catalyzed crosslinking) on the immune response in late-lactating dairy goats. Dairy goats (n = 18) were randomly assigned to three experimental groups (n = 6). Dairy goats were orally administered 0.35 mg of vitamin D_3/day in the unencapsulated form and 0.35 mg of vitamin D_3/day in the encapsulated powder form. Another group received the basal diet. The experimental period lasted 6 weeks. The blood serum concentrations of 25-hydroxyvitamin D_3 [25-(OH)-D_3], lactoferrin, immunoglobulin A (IgA), and interferon-gamma (INF-γ) were measured. There were major differences in these parameters between dietary groups. However, the delivery of vitamin D_3 in the encapsulated powder form to dairy goats resulted in a marked increase in 25-(OH)-D_3 concentration in serum, while the serum level of lactoferrin also increased. Alternatively, the serum levels of IgA and the immunomodulatory cytokine INF-γ were elevated following supplementation with the encapsulated vitamin D_3. The observed effects suggest that the deliverable form of dietary vitamin D_3 results in differences in the immune response in late-lactating dairy goats.

Keywords: bovine lactoferrin; complex coacervation; dairy goats; encapsulation; immune response; sodium alginate; vitamin D_3

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1. Introduction

Vitamin D_3, which is formed in the skin or absorbed through the gastrointestinal tract, is modified by enzymatic hydroxylation to form first in the liver, 25-(OH)-D_3, which is a plasma transport form of the vitamin, and then in the kidney to form 1,25-$(OH)_2D_3$, the active hormone, or 24,25-$(OH)_2D_3$ form of the vitamin and other derivatives. It appears that vitamin D_3 stimulates the active transport of calcium across mucosal cells of the small intestine [1]. In growing dairy goats (*Capra hircus*) and sheep (*Ovis aries*), a vitamin D_3-reduced diet combined with missing ultraviolet B (UVB) exposure leads to a sharp drop in the vitamin D_3 status [2]. Such findings reinforce the results of studies that employed vitamin D_3 supplementation and UVB exposure in juvenile bearded dragons (*Pogona vitticeps*) [3]. The study reported by Oonincx et al. [3] showed that 2 h of UVB exposure enables adequate physiological concentrations of plasma vitamin D_3 metabolites to be maintained in growing bearded dragons. Vitamin D_3 also has a precise role in immunoregulation in response to microbial threats [4]. The protective effect of vitamin D_3

on infectious diseases such as SARS-CoV-2 (COVID-19), tuberculosis, and mastitis has been linked to a direct interaction of vitamin D_3 with cells of the immune system [4–6].

Vitamin D_3 is often included in foods as dispersions in emulsified lipid carriers (e.g., milk), which provide an effective barrier against environmental stresses (e.g., oxidation). A variety of delivery systems have been developed to encapsulate bioactive compounds, and most are applicable to the encapsulation of lipid-soluble bioactive compounds [7].

Usually, encapsulation allows for an even dispersion of bioactive compounds throughout the products, meaning consumers will receive the same amount of a bioactive compound, such as vitamin D_3, in each bite of food or sip of a drink [8]. Encapsulation also improves the shelf-life of products, thereby preventing vitamin D_3 from degrading over time and keeping the formulation stable [8]. There are also concerns about intestinal absorption of liposoluble bioactive compounds. Properly encapsulated liposoluble bioactive compounds such as vitamin D_3 are thought to enter the bloodstream faster and in greater amounts [8] compared to the unencapsulated forms.

Protein-polysaccharide complex coacervates can be used as inexpensive encapsulation carriers for bioactive compounds in food and beverage products [9]. The coacervation encapsulation approach involves the processing steps of emulsification, coacervation, and shell formation and/or hardening [10]. Coacervation is a chemical method for producing biopolymer droplets in suspension based on the separation of two liquid phases into one concentrated colloidal phase [11]. Complex coacervation is the result of electrostatic interaction between two biopolymers with opposite charges, generally a protein and a polysaccharide [10]. Complex coacervates between whey proteins (e.g., bovine lactoferrin) and polysaccharides such as carboxymethylcellulose, pectin, and alginate have been recently used to encapsulate liposoluble bioactive compounds [12,13].

The use of cross-linking agents provides more resistant structures to capsules produced by colloidal complexation [14] or complex coacervation [15]. The coacervation encapsulation process may increase the absorption of bioactive compounds through controlled release [16]. A previous study reported that the polysaccharide sodium alginate has promising properties to be used as a carrier with adjuvants and prolong release properties that enhance the immunogenicity of vaccines [17]. Thus, guinea pigs immunized with diphtheria toxoid-loaded alginate nanoparticles showed the highest humoral immune response to the conventional vaccine [17]. Moreover, bovine lactoferrin may be a potential alternative therapy or may serve as an adjunct to conventional therapies for the treatment of inflammation [18]. In this context, bovine lactoferrin has been shown to have good activity against inflammation in vitro, with systemic effects suggested for various inflammatory diseases by stopping the production of pro-inflammatory molecules [18]. Recently, more evidence has been rising for the direct effects of bovine lactoferrin on the immune system [19].

The primary aim of this study was to specifically evaluate the immunomodulatory role of vitamin D_3 encapsulated by sulfur-saturated bovine lactoferrin-alginate complex coacervates using microbial transglutaminase as a cross-linking agent in late-lactating dairy goats. Ingestion of vitamin D_3 capsules may result in a marked and rapid increase in immunological responses for the prevention of bacterial invasion (e.g., mastitis) in the mammalian host. The unencapsulated form of vitamin D_3 was used for comparison purposes.

2. Materials and Methods

Cholecalciferol (vitamin D_3), dimethyl sulfone ($DMSO_2$), sodium alginate, and microbial transglutaminase were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 25-hydroxyvitamin D_3 [25-(OH)-D_3] was purchased in crystalline form from Cayman Chemical (Ann Arbor, MI, USA). Medium-chain triglycerides (MCTs) oil (Neobee M-5, $\geq$66% C8:0 and $\geq$32% C10:0 content) was kindly donated by Stepan Company (Northfield, IL, USA). Bovine lactoferrin-FD (10321412) was a gift from MDV International (Delhi, NY, USA), whose purity was more than 90% and 15.7 mg of iron/100 g of bovine lactoferrin. Bovine lactoferrin (2 g) was dissolved in 0.1 M sodium bicarbonate with dimethyl sulfone

(0.5 g) for 24 h at room temperature (20 °C), followed by extensive dialysis against sodium bicarbonate to remove excess sulfur ions, and then the solution was freeze-dried (LABCONCO, Kansas City, MO, USA). Sulfur saturation of bovine lactoferrin with dimethyl sulfone was 90.4% as determined by the MACRO Cube Elemental Analyzer (Quantum Analytics, The Woodlands, TX, USA). Sulfur nanoparticles have shown antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [20]. Bovine lactoferrin *per se* is active against mastitis pathogens, but its antimicrobial activity is low enough to constitute a viable treatment against mastitis pathogens [21]. However, the efficacy of bovine lactoferrin against pathogenic bacteria and viruses can be increased by the metal saturation of lactoferrin [22]. The solvents used for the extraction and analysis of samples were HPLC-grade and purchased from Sigma-Aldrich. All other chemicals and reagents were of analytical-grade (Sigma-Aldrich). Deionized water, prepared by passing distilled water over a mixed-bed cation-anion exchanger, was used throughout this study.

The protein and polysaccharide were prepared to contain a total concentration of 1.0% (*w*/*w*) and a biopolymer mixing ratio of 9:1 (sulfur-saturated bovine lactoferrin:sodium alginate) in an aqueous solution at pH 4.0. Vitamin D_3, previously dispersed in warmed (37 °C) MCTs oil (0.75%, *w*/*w*) by a magnetic stirring hotplate (Thermo Fisher Scientific, Waltham, MA, USA), was added to the aqueous solution (74.25%, *w*/*w* in the final emulsion) of sulfur-saturated bovine lactoferrin to give a concentration of 540 µg/mL vitamin D_3 in the final emulsion, and pre-emulsified with a hand-held homogenizer (Biospec Products Inc., Bartlesville, OK, USA) at low speed for 3 min at 20 °C. The sodium alginate aqueous solution (25.0%, *w*/*w* in the final emulsion), previously acidified to pH 4.0 with acetic acid (20%, *w*/*w*), and the coarse emulsion were homogenized twice at 82.74 MPa (12,000 psi) at a temperature of 50 °C through a high-pressure TC5 homogenizer (Stansted Fluid Power, Harlow, UK). The temperature of the oil-in-water emulsion was reduced to 5 °C using an ice-water bath for 60 min. Microbial transglutaminase solution (0.25%, *w*/*w*) was added under constant magnetic stirring (Thermo Fisher Scientific) at 400 rpm for 3 h at 25 °C to induce cross-linking. Immediately after treatment, microbial transglutaminase was inactivated by freezing the oil-in-water emulsion for 10 min. The vitamin D_3 capsules were kept at 10 °C for 48 h, and then the supernatant was removed by decantation. The vitamin D_3 capsules were frozen in liquid nitrogen and freeze-dried for 24 h. The quantitative determination of vitamin D_3-loaded capsules was performed using HPLC-UV [23].

The encapsulation efficiency (EE%) of the vitamin D_3 capsules was calculated as follows:

$$\text{EE\%} = (\text{loaded vitamin } D_3/\text{total vitamin } D_3) \times 100 \quad (1)$$

The stability of vitamin D_3-loaded capsules was evaluated during storage at 24 °C under nitrogen gas for 6 months. Vitamin D_3 content was measured at the 1st, 5th, 10th, 15th, 30th, 60th, 90th, and 180th days. The content of vitamin D_3 was determined by HPLC-UV [23].

In a 6-week feeding trial, eighteen American-bred, French-Alpine dairy goats (mean age 2.5 years, mean BW 114.2 kg) were selected from the milking herd of the International Goat Research Center (IGRC) at Prairie View A&M University, Prairie View, TX, USA. All experimental procedures with the goats were in strict compliance with the current guidelines and legal requirements established in the United States for the proper use and care of animals and approved by the Institutional Animal Care and Use Committee at Prairie View A&M University (Protocol # 2023-051). A total of six goats per experimental group (n = 18) were used. Our sample size also follows the recommendations of the Institutional Animal Care and Use Committee at Prairie View A&M University for discovery experiments where at least 4 biological replicates are needed.

All dairy goats were housed indoors at the IGRC. The temperature in the house was approximately 25 °C. The selected dairy goats were randomly assigned to three experimental groups: control (n = 6), vitamin D_3 supplementation (0.35 mg vitamin D_3/day) in the unencapsulated form previously dispersed in MCTs oil (n = 6), and vitamin D_3 supplementation (0.35 mg vitamin D_3/day) in the encapsulated powder form (n = 6). After

the initial 2 weeks of adjustment period, the experiment continued for 6 weeks. All goats were in late lactation [172 DIM; 1.06 L/day; level of α_{s1}-casein (high: 18.8% of total protein)] and randomly assigned to an individual feeding gate on the day of enrollment.

Goat's α_{s1}-casein milks produced by animals of Alpine breed in the same stage of lactation used in this study were confirmed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS), and densitometry was used to assess the relative concentration of the α_{s1}-casein component [24].

The treatment of dairy goats fed the encapsulated vitamin D_3 consisted of 1 g of vitamin D_3 capsules per goat (0.35 mg vitamin D_3/day) in addition to the base ration that provided 0.05 mg of vitamin D_3 daily. As a functional protein, sulfur-saturated bovine lactoferrin is an interesting biopolymeric wall system used to encapsulate vitamin D_3. Thus, the treatment of the group of dairy goats fed the encapsulated vitamin D_3 consisted of 1 g of the vitamin D_3 capsules that provided sulfur-saturated bovine lactoferrin (900 mg sulfur-saturated bovine lactoferrin/day). It should be noted here that the treatment group of dairy goats fed the encapsulated vitamin D_3 consisted of 1 g of vitamin D_3 and also provided a polysaccharide, sodium alginate (100 mg sodium alginate/day), used to encapsulate vitamin D_3. A top-dress supplement for the treatment with the encapsulated vitamin D_3 was prepared by mixing 1 g of the vitamin D_3 capsules with cornmeal to provide 0.35 mg of vitamin D_3, 900 mg of sulfur-saturated bovine lactoferrin, and 100 mg of sodium alginate in 100 g of the total mixture. A top-dress supplement for the treatment with the unencapsulated vitamin D_3 was prepared by combining vitamin D_3 in MCTs oil (0.35 mg of vitamin D_3/5 mL of MCTs oil), homogenized for adequate dispersal, and mixed with cornmeal to provide 0.35 mg of vitamin D_3 in 100 g of the total mixture. The two top-dress supplements were not mixed into the ration and were consumed readily by all goats upon delivery.

The control animals received the basal diet without supplementation. The basal diet was composed of 69.3% oats, 1.35% soybean meal, 2.05% corn, 26.04% alfalfa meal, 0.93% cottonseed hulls, 0.31% dicalcium phosphate, and 0.06% vitamin A, D_3, and E supplements. The experimental and control animals were fed twice a day, in the morning and the evening. The animals were given 2.07 kg of either the control or the experimental diet twice a day, in the morning and evening, and the leftovers were measured. The unencapsulated vitamin D_3 and the encapsulated vitamin D_3 were only added to the morning feeding as a topdress. The feeding duration of this experiment was 6 weeks. Hay and water were available to animals *ad libitum*. Housing and care of the animals conformed to the approved institutional animal care and use committee practices and standards of the university.

Blood samples (5 mL) were collected by puncture of the jugular vein using siliconized needles (21 G $\times$ 1 in) with a vacuum system. The blood samples taken at 0 and 6 weeks of treatment were transferred into a 10-mLvacuum tube (serum separator tube) (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Tubes were centrifuged at 3500$\times$ *g* for 5 min in a refrigerated centrifuge (4 °C) for serum separation within 30 min of sample collection. Serum samples were transferred into microtubes using Pasteur pipettes. The samples were maintained at 2–8 °C and immediately analyzed, avoiding freeze–thaw cycles because this is detrimental to many serum components.

All serum analyses were performed in triplicate. Serum 25-(OH)-D_3 concentrations were quantified using a CDC-certified LC-MS/MS method [25]. Serum lactoferrin, IgA, and INF-γ concentrations were quantified using commercially available ELISA kits (Sigma-Aldrich), according to the manufacturer's directions.

Statistical Analysis

Quantitative data for the combination of response and treatment variables are summarized with a mean $\pm$ standard error. A paired *t*-test under the pre-posttest design was performed to compare the difference between before supplementation (week 0) and after supplementation (week 6) with animals assigned to different dietary groups. The outcome variables [25-(OH)-D_3, lactoferrin, IgA, and INF-γ serum concentrations] were measured

before and after the supplementation. The 5% significance level was applied to the paired *t*-test. Experimental data were analyzed using SAS software (version 9.4, SAS Institute, Cary, NC, USA).

3. Results and Discussion

The encapsulation of vitamin D_3 attained a high encapsulation efficiency of 91.2%. The capsules contained vitamin D_3 at a concentration of 0.35 mg per gram (0.035%) as determined by HPLC-UV [23]. As to the storage stability of the vitamin D_3 capsules, there was little change in concentration after 3 months of storage at 24 °C, with a decrease in concentration of about 0.2%. Six months later, the storage stability decreased by 1.3% at 24 °C, which was not significant. These results suggest that vitamin D_3 loading in the complex coacervates of sulfur-saturated lactoferrin and sodium alginate with transglutaminase, as an effective protein cross-linker, has good stability.

Studies involving different species of experimental animals and using deficient as well as supradietary levels of vitamin D_3 indicate that the vitamin D_3 metabolite 25-(OH)-D_3 is involved in the maintenance of immune function [26,27]. Activated T lymphocytes and macrophages, among others, have been found to express vitamin D receptors [28]. Several clinical trials have demonstrated the potential benefit of increased vitamin D status on immune function [29]. In these clinical trials, the supplemented groups showed significant improvement in several parameters of immune responsiveness and a marked reduction in illness due to infections; moreover, supplemented subjects presenting with illness were observed to require shorter antibiotic treatment regimens.

The *in vivo* test performed on dairy goats confirmed the efficiency of the intake of encapsulated vitamin D_3 (0.35 mg vitamin D_3/day) in increasing the concentration of circulating 25-(OH)-D_3 during late lactation (Table 1). Charoenngam and Holick [30] noted that enhancing 25-(OH)-D_3 status contributes to increased immunological vigor in humans. Merriman et al. [31] found that intramammary 25-(OH)-D_3 treatment directly influences immune cells of the mammary gland of lactating dairy cows in response to endotoxin-induced mastitis by increasing vitamin D signaling in mammary immune cells. Moreover, Poindexter et al. [32] observed that feeding 25-(OH)-D_3 to lactating dairy cows increased circulating 25-(OH)-D_3, which appears to improve immune responses to the intramammary *Streptococcus uberis* challenge, thereby protecting the mammary glands from mastitis. These nutritionally induced changes observed by Poindexter et al. [32] also occurred *via* vitamin D signaling. Comparison of 25-hydroxyvitamin D_3 [25-(OH)-D_3] serum levels between baseline and supplementation (unencapsulated vitamin D_3 vs. encapsulated vitamin D_3) indicated significant ($p < 0.0001$) improvements in both supplemented groups (Table 1), but we observed a higher ($p < 0.0001$) 25-(OH)-D_3 serum concentration at 6 weeks in dairy goats fed the encapsulated form of vitamin D_3, compared with the serum concentration of dairy goats fed the unencapsulated form of vitamin D_3 at 6 weeks (Table 1). This may be attributable to the efficacy of the delivery system.

The form in which vitamin D_3 is delivered can have a great influence on whether that vitamin D_3 is consumed and could influence the immunomodulatory effects of vitamin D_3. One must consider dosage factors to equate for body weight, which leads to different serum levels of vitamin D_3 metabolites. One must also consider the effects of milk production during late lactation and milk quality in terms of technological properties, where high-type α_{s1}-casein milk seems more suitable for cheesemaking because of its firmer curd and higher casein content. Given all these variables, and perhaps others as yet unknown, it is not surprising that the effects of vitamin D_3 in the unencapsulated and encapsulated forms are seen as highly individual. Vitamin D_3, in unencapsulated form, at 0.35 mg/day was provided for 6 weeks, followed by testing. Although, surprisingly, the dose did not result in significant differences in serum levels of lactoferrin, IgA, and INF-γ, a clearer dose response was seen in serum levels of 25-(OH)-D_3 in the unencapsulated group of dairy goats (Table 1).

Table 1. Serum concentrations showing significant differences in 25-hydroxyvitamin D_3 [25-(OH)-D_3], lactoferrin, immunoglobulin A (IgA), and interferon-gamma (INF-γ) at baseline (0 week) and at feeding period (6 weeks) between dietary groups [1,2].

Item	Baseline (0 Weeks)	Feeding Period (6 Weeks)	*p*-Value
25-(OH)-D_3 (ng/mL)			
Treatment U	25.92 ± 1.63	39.10 ± 1.53	<0.0001
Treatment E	27.30 ± 0.93	51.37 ± 1.27	<0.0001
Control	23.53 ± 0.78	24.35 ± 1.29	0.500
Lactoferrin (μg/mL)			
Treatment U	148.28 ± 1.03	149.60 ± 3.51	0.728
Treatment E	146.90 ± 1.32	228.40 ± 4.54	<0.0001
Control	147.08 ± 1.27	146.60 ± 0.75	0.723
IgA (μg/mL)			
Treatment U	430.12 ± 3.57	425.27 ± 5.10	0.547
Treatment E	427.72 ± 5.70	576.62 ± 2.01	<0.0001
Control	428.15 ± 4.75	426.61 ± 1.83	0.763
INF-γ (pg/mL)			
Treatment U	36.33 ± 3.48	39.23 ± 4.13	0.701
Treatment E	35.17 ± 2.49	62.08 ± 2.00	<0.0001
Control	32.27 ± 2.40	36.81 ± 2.16	0.807

[1] Dairy goats in late lactation fed a basal diet (control group), a basal diet supplemented with the unencapsulated form of vitamin D_3 (treatment group U), and a basal diet supplemented with the encapsulated form of vitamin D_3 (treatment group E). [2] Data are presented as means ± standard error; n = 18.

We also observed that supplementation with the encapsulated vitamin D_3 significantly ($p < 0.0001$) increased the serum levels of lactoferrin compared to the group of dairy goats that were fed the unencapsulated vitamin D_3 (Table 1). The sulfur-saturated bovine lactoferrin, was used as a biopolymeric wall system to encapsulate vitamin D_3, which contributes to immune regulation in lactating dairy goats by maintaining the circulation of IgA in late lactation at a high concentration compared to the experimental group of dairy goats fed the unencapsulated vitamin D_3 (Table 1). Lactoferrin should, in theory, exert an advantageous immunomodulatory effect because it can pass through the blood/milk barrier, thereby protecting the mammary gland from bacterial invasion [33]. In the experimental group of dairy goats that were fed the encapsulated vitamin D_3, the serum levels of IgA were significantly ($p < 0.0001$) increased (Table 1). Lactoferrin has been identified as a critical nutrient, and the combination of lactoferrin and IgA is particularly important for infants, whose immune systems are still developing. Breast milk contains high levels of both lactoferrin and IgA, which help to protect infants against infections [34].

As noted, 25-(OH)-D_3 is a precursor to the active form of vitamin D_3, which is a hormone that plays a vital role in many bodily functions, including immune regulation [35]. The oral intake of vitamin D_3 or preformed vitamin D_3 [25-(OH)-D_3] has been shown to work together with lactoferrin to boost immune function even more effectively than either one alone among those individuals who were affected by exposure to SARS-CoV-2 infection [36]. Moreover, high serum levels of INF-γ, an immunomodulatory cytokine, are associated with a stronger immune response and improved protection against infection [37]. INF-γ production was markedly higher ($p < 0.0001$) in blood serums obtained from the dairy goats fed the encapsulated vitamin D_3 (0.35 mg vitamin D_3/day) than from the group of dairy goats fed the unencapsulated vitamin D_3 (0.35 mg vitamin D_3/day) (Table 1). These results suggest that the encapsulated vitamin D_3 exhibits sustained release and enhanced absorption through complex coacervation technology.

4. Conclusions

These vitamin D_3 capsules can be used as dietary supplements for their beneficial effects on the host and its immune system.

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Institutional Review Board Statement: The study was conducted in strict compliance with the current guidelines and legal requirements established in the United States for the proper use and care of animals and approved by the Institutional Animal Care and Use Committee of Prairie View A&M University (Protocol # 2023-051).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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