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Impact of Feeding Vitamin D_3 Encapsulated by Sulfur-Saturated Bovine Lactoferrin-Alginate Complex Coacervates Using Microbial Transglutaminase on the Immune Response of Late-Lactating Dairy Goats

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Abstract: Mastitis-causing bacteria can establish persistent infections in the mammary gland of commercially important dairy animals despite the presence of strong specific humoral and cellular immune mechanisms. The aim of this study was to encapsulate vitamin D_3 by complex coacervation with sulfur-saturated bovine lactoferrin-alginate using microbial transglutaminase-catalyzed crosslinking and investigate the oral administration of encapsulated vitamin D_3 in blood serum biomarkers of the immune response in late-lactating dairy goats. Dairy goats (n = 18) in late lactation were randomly assigned to three experimental groups (n = 6), after balancing weight. Dairy goats were orally administered with 0.35 mg vitamin D_3/day in the unencapsulated form and 0.35 mg 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. The delivery of vitamin D_3 to dairy goats resulted in a marked increase of 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. Overall, the capsules in the powder form used in this study kept vitamin D_3 highly bioavailable.

Keywords: vitamin D_3; sulfur-saturated bovine lactoferrin-alginate complex coacervates; microbial transglutaminase; immune response; late-lactating dairy goats

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 active transport of calcium across mucosal cells of the small intestine [1]. Vitamin D_3 also has a precise role in immunoregulation in response to microbial threat [2]. 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 on cells of the immune system [3–5].

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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 [6].

Usually, encapsulation allows for even dispersion of bioactive compounds throughout the products, meaning consumers will get the same amount of a bioactive compound, such as vitamin D_3 in each bite of food or sip of a drink [7]. Encapsulation also improves the shelf-life of products, thereby preventing vitamin D_3 from degrading over time and keeping the formulation stable [7]. There are also the concerns of 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 [7] compared to the unencapsulated forms.

Protein-polysaccharide complex coacervates can be used as inexpensive encapsulation carriers for bioactive compounds in food and beverage products [8]. The coacervation encapsulation approach involves the processing steps of emulsification, coacervation, and shell formation and/or hardening [9]. Coacervation is a chemical method for producing biopolymer droplets in suspension based on the separation of two liquid phases into one concentrated colloidal phase [10]. Complex coacervation is the result of electrostatic interaction between two biopolymers with opposite charges, generally a protein and a polysaccharide [9]. 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 [11,12].

The use of cross-linking agents provides more resistant structures to capsules produced by colloidal complexation [13] or complex coacervation [14]. The coacervation encapsulation process may increase the absorption of bioactive compounds by controlled release [15].

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 in late-lactating dairy goats. Ingestion of the 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.

2. Materials and Methods

Cholecalciferol (vitamin D_3), methylsulfonylmethane, 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, ≥ 66% C8:0 and ≥ 32% C10:0 content) was kindly donated by Stepan Company (Nortfield, 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 methylsulfonylmethane (0.5 g) for 24 h at room temperature (20 °C), followed by extensive dialysis against sodium bicarbonate to remove 'unbound' sulfur ions and then the solution was freeze-dried (LABCONCO, Kansas City, MO, USA). Sulfur saturation of bovine lactoferrin with methylsulfonylmethane was 90.4% as determined by MACRO Cube Elemental Analyzer (Quantum Analytics, The Woodlands, TX, USA). 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 biopolymer mixing ratio of 9:1 (sulfur-saturated bovine lactoferrin:sodium alginate) in 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), 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

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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 was 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, U.K). 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 [16].

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

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

The stability of vitamin D_3-loaded capsules was evaluated during storage at 24 °C under nitrogen gas for a period of 6 months. Vitamin D_3 content was measured at 1st, 5th, 10th, 15th days, 30th days, 60th days, 90 days, and 180 days. The content of vitamin D_3 was determined by HPLC-UV [16].

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 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.

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) and goats were randomly assigned to an individual feeding gate on the day of enrollment.

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 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 vitamin D_3 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 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 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 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 supplement. The experimental and the control animals were fed twice a day, in the morning and in the evenings. 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

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leftovers were measured. The unencapsulated vitamin D_3 and the encapsulated vitamin D_3 were only added to the morning feeding as a top-dress. 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 x 1 in) with a vacuum system. The blood samples taken at 0 week and 6 weeks of treatment were transferred into 10-mLvacuum tube (serum separator tube) (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Tubes were centrifuged at 3,500X *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, which has been described in detail elsewhere [17]. 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 variable are summarized with mean ± standard error. A paired *t* test under 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] are 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

After loading vitamin D_3 within the sulfur-saturated bovine lactoferrin-sodium alginate complex coacervates followed by transglutaminase crosslinking, the physicochemical properties of the encapsulated vitamin D_3 delivery system such as encapsulation efficiency and storage stability were evaluated. 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 [16]. 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 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, have good stability.

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 in 2020 [18] noted that enhancing 25-(OH)-D_3 status contributes to increased immunological vigor in humans. Merriman et al. in 2018 [19] 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_3 signaling in mammary immune cells. Moreover, Poindexter et al. in 2020 [20] observed that feeding 25-(OH)-D_3 to lactating dairy cows increased circulating 25-(OH)-D_3, which appears to improve immune responses to intramammary *Streptococcus uberis* challenge, thereby protecting the mammary glands from mastitis. These nutritionally-induced changes observed by Poindexter et al. in 2020 [20] also occurred *via* vitamin D_3 signaling. A dietary requirement for a source of preformed vitamin D_3 (e.g., [25-(OH)-D_3]) has been identified by Poindexter et al. in 2020 [20] as necessary for normal development of cellular immune responses of lactating dairy cows. 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 ($p < 0.0001$)

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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.

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 week)	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 mean ± standard error; n = 18.

Human lactoferrin is a natural whey protein that helps to fight and prevent infections and strengthens the body's defense system [21]. This whey protein is present in substantial quantities in mother's milk and plays an important role in the defense system of infants. This whey protein is also present in various body fluids and continues to play an important role against a wide range of infections [21]. Likewise, the consumption of bovine lactoferrin has been shown to support the immune and antioxidant status of healthy human males [22]. Studies in mice have demonstrated that the oral use of bovine lactoferrin increases immune function and resistance to infection [23]. In our study, we were looking at vitamin D_3 (0.35 mg vitamin D_3/day) encapsulated with sulfur-saturated bovine lactoferrin (900 mg sulfur-saturated bovine lactoferrin/day) and sodium alginate (100 mg sodium alginate/day) to see if it could enhance the immune response of dairy goats at late-lactating stages. We observed that supplementation with the encapsulated vitamin D_3 significantly ($p < 0.0001$) increases 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 that contributes to immune regulation in lactating dairy goats by maintaining the circulation of IgA in late lactation at high concentration compared to the experimental group of dairy goats fed the unencapsulated vitamin D_3 (Table 1). Lactoferrin should in theory exert advantageous immunomodulatory effect because it can pass through the blood/milk barrier, thereby protecting the mammary gland from bacterial invasion [24].

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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 [25].

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 [26]. 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 [27]. Moreover, high serum levels of INF-γ, an immunomodulatory cytokine, are associated with a stronger immune response and improved protection against infection [28]. 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). This observation suggests that the consumption of encapsulated vitamin D_3 may be associated with an improved immune defense.

4. Conclusions

Hence, we are reporting evidence that an increased uptake of vitamin D_3 in late-lactating dairy goats supplemented with the encapsulated vitamin D_3 has occurred. We have shown that a total vitamin D_3 intake of 0.35 mg vitamin D_3/day as encapsulated vitamin D_3 is sufficient to maintain 25-(OH)-D_3 concentrations > 50 ng/mL. Additionally, serum levels of IgA and INF-γ were also significantly ($p < 0.0001$) increased. We speculate that the sulfur-saturated bovine lactoferrin (900 mg sulfur-saturated bovine lactoferrin/day) used in this study as a biopolymer to encapsulate vitamin D_3 stimulates IgA release in serum. Such sulfur-saturated bovine lactoferrin works synergistically with serum 25-(OH)-D_3 to boost the immune response as evidenced by the high serum levels of INF-γ. Our data, apart from indicating bioavailability to vitamin D_3, indicates that sulfur-saturated lactoferrin is a possible alternative for commercial bovine lactoferrin in freeze-dried lactoferrin-polysaccharide systems.

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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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Conflicts of Interest: The authors declare no conflicts of interest.

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