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Article

Stabilization of Vitamin D in Pea Protein Isolate Nanoemulsions Increases its bioefficacy in Rats

Ali M. Almajwal, [1]* Mahmoud M.A. Abulmeaty, [1] Hao Feng, [2] Nawaf W. Alruwaili, [2] Astrid Dominguez-Uscanga,[2] Juan E. Andrade [2]*, Suhail Razak,[1,] and Mohamed F. ElSadek [2]

[1] Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia; aalmajwal@ksu.edu.sa, mabulmeaty@ksu.edu.sa, smarazi@ksu.edu.sa, mfbadr@ksu.edu.sa

[2] Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, USA, haofeng@illinois.edu, alruwai2@illinois.edu, astrid86@illinois.edu, jandrade@illinois.edu

* Correspondence: aalmajwal@ksu.edu.sa; Tel.: + 966 1 4693699

Abstract: 1) Background: The aim was to evaluate the bioefficacy of vitamin D (VitD) encapsulated in nanoemulsions developed by sonication and pH-shifting of pea protein isolate in restoring VitD status in VitD-deficient rats. 2) Methods: Weaned (3-week old), male albino rats (n=35) were initially divided into two groups: control sufficient group (VDS; n=7) fed on a normal AIN-93G diet and a VitD-deficient group (n=28) fed a VitD-deficient diet for six weeks. VitD-deficient rats were divided into four subgroups: two treatment groups (Nano+VitD and Oil+VitD) and two control groups (Nano-VitD and Oil-VitD), receiving seven rats in each sub-group. Nano+VitD and Oil+VitD groups received VitD dispersed in PPI-nanoemulsions and in canola oil, respectively, while the control groups received the respective delivery vehicles without VitD. Serum 25-hydroxyvitamin D [25(OH)VitD], parathyroid hormone (PTH), calcium (Ca), phosphorus levels (P), and alkaline phosphatase (ALP) activity were measured. Femur bone was used to prepare histopathological sections. 3) Results: After one week of treatment, the VitD-deficient rats consuming Nano+VitD recovered from VitD deficiency (serum 25(OH)VitD 34.38±7.00) compared to the sufficient control (36.84±9.16; $P>0.05$) and the deficient control consuming VitD+Oil (14.05±3.08; $p<0.01$). Enhancement in VitD status was followed with expected changes in serum PTH, Ca, P, and ALP levels, no difference in similar biomarkers against the sufficient control, and an improvement of the osteoid area and reduction of trabecular separation in bone. 4) Conclusions: Stabilization of VitD within PPI-based nanoemulsions enhances its absorption and restores its status and biomarkers of bone resorption in VitD-deficient rats.

Keywords: Nanoemulsion, Vitamin D, vitamin D deficiency, bioefficacy, fortification, rat

1. Introduction

Vitamin D (VitD) is a fat-soluble vitamin derived from skin production through exposure to ultraviolet light and from food intake. VitD is used by the body for normal bone development and maintenance by increasing the absorption of calcium, magnesium, and phosphorus [1]. VitD from the skin and diet is metabolized in the liver to 25-hydroxyvitamin D (25(OH)VitD), which represents a total concentration of both 25-hydroxyvitamin D2 (from plant ergocalciferol) and 25-hydroxyvitamin D (from cholecalciferol). Serum 25(OH)VitD is used as a biomarker of VitD status. Circulating levels of 25(OH)VitD greater than 20 ng/mL (50 nmol/L) indicate sufficient VitD status,

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whereas levels below 12 ng/mL are considered deficient. Although under debate, 25(OH)VitD levels between 12 and 20 ng/mL are considered insufficient [2,3].

Causes of vitamin D deficiency (VDD) include an inadequate dietary intake and limited exposure to sunlight. About 50% to 90% of vitamin D is absorbed through the skin via sunlight while the rest comes from the diet. Severe VDD leads to rickets in children and osteomalacia in adults. In adults, it also predisposes to low bone mass and contributes to bone fragility fractures in the elderly [4]. In recent years, there has been a renaissance in the study of VitD actions as evidence continues to accumulate about its role in the etiology of chronic disease such as infection response, autoimmune disease, cardiovascular disease, diabetes mellitus, and cancer [5].

Prevention of VDD remains a global health priority [6]. Food fortification with VitD is considered one of the most cost-effective strategies to combat VDD. In this regard, much of the attention has been given in developing sound delivery systems to carry VitD efficiently and safely at all bio-physiological levels in the body [7]. In the last few years, lipids [8–11] and polymers [12–14] have been extensively investigated, used and reevaluated as prospective VitD delivery systems.

There are certain limitations associated with these delivery systems such as short half-life, susceptibility to oxidation, possibility of hydrolysis, leakage and fusion, allergenicity, and relatively high cost of scaling up the process to achieve a reproducible, high quality products [15]. These limitations make such delivery systems less attractive to the food industry [16]. In addition to the mentioned limitations, there are some health risks associated with the consumption of cationic polymers, especially when administered at high concentrations [17]. Hence, a potential delivery system must be harmless for both the intended users (animals or humans) and the environment, in which its implementation results in minimum health risks and maximum cost-effectiveness.

One possible way to achieve this goal is by using food proteins; for example, by binding VitD to the β-lactoglobulin from whey, which is a by-product of cheese making [18–20]. Diarrassouba et al., (2015) showed an alternative 'green method', which uses oppositely charged food proteins egg lysozyme and β-lactoglobulin resulting in protein-based microspheres with high VitD encapsulation efficiency. Most of these methods, however, involve tedious extraction procedures, gradient flows, long elution times, and often a purification step before their estimation. Moreover, several of these methods utilize solid phase extraction processes involving high processing cost [21,22].

Plant proteins are of particular interest as emulsifiers in food systems mainly due to their ability to adsorb to oil-water interfaces and form interfacial films [23,24]. The surface activity of proteins is the result of their amphiphilic nature, because of the presence of both hydrophobic and hydrophilic regions in their peptide chains [25]. Legume proteins are gaining popularity for this purpose due to their high natural abundance, sustainability, low cost, and functional attributes [26]. The main objective of this study was to assess the ability of novel protein-based nanoemulsions created from the sonication and pH-shifting of pea protein isolate (PPI) in enhancing VitD bioefficacy as evaluated in a rodent model of VitD deficiency.

2. Materials and Methods

2.1. Materials

The following materials were obtained for all experiments. Pea protein isolate (PPI, NUTRALYS® S85F, 85% pea protein based on dry basis) in powder form was provided by Roquette (Geneva, IL, USA), and was produced using a wet extraction process from dry yellow peas. PPI was kept at 4 °C before use. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA) and were of analytical or higher grade.

2.2 Formation of pea protein nanoaggregates

The method of Jiang et al. (2017) was followed [27]. Briefly, pea protein nanoaggregates were prepared by adding PPI (3 g) into a beaker containing 100 mL double deionized water followed by stirring for 30 min at room temperature (23 °C). PPI dispersion was adjusted to pH 12 with a few drops of 2M NaOH. Ultrasound treatment was applied for 5 minutes using a VC-750 ultrasonic

processor at 20 kHz (Sonics & Materials, Inc., Newtown, CT, USA). Acoustic energy was delivered to the center of the dispersion using a probe (12.5 mm diameter). Excessive heating was controlled by placing the sample on an iced water bath. After ultrasound treatment, samples were held for 1 h at room temperature before adjusting to pH 7 with a few drops of 2M HCl. The neutralized PPI nanoaggregate dispersion was centrifuged for 15 min (Sorvall Instruments RC5C, Rotor GSA code 10, Newton, CT) at 8,610 RPM, 15 °C. The supernatant was collected and stored to create protein nanoemulsions.

2.3 Preparation of nanoemulsion

VitD-containing nanoemulsions were prepared by homogenizing 0.4% VitD (cholecalciferol, 98% pure) at a fixed concentration (2% w/v) of PPI-nanoaggregates as the emulsifier. Both phases were stirred for 5 min followed by ultrasound (5 min) similar as above. Samples were cooled and the final concentration as measured by HPLC-RP [27] was 27 µg VitD/mL.

2.4 Animals

All experiments with rats were conducted in the Animal Laboratory located in the College of Applied Medical Sciences (CAMS) at King Saud University. Research principles and ethical guidelines of the KSU-CAMS Research Ethics Committee were strictly observed for all experiments using animals. Thirty-five healthy, male Wistar rats were procured from Animal Research Center, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia. After weaning for 3 weeks, rats were divided primarily into two groups: the VitD-sufficient (VDS group) and VitD-deficient group. The rats in the VDS group (n=7) were fed on normal balanced AIN-93G diet (protein 18.1%, fat 7.1%, carbohydrates 59.3%, fiber 4.8%, ash 2.2%, calcium 5.1 g/kg, phosphorus 2.8g/kg, and vitamin D 1000 IU/kg) [28] throughout the study. This diet contained 1,000 IU (25 µg) of cholecalciferol per kg. The VitD-deficient rats (n=28) were fed a customized VitD-deficient AIN-93G diet (VitD <50 IU) with normal Ca and P for six-weeks [29]. After six-weeks, VitD-deficient rats were divided into four distinct subgroups. The treatment groups received 3 mL of 27 µg/mL VitD stably dispersed in nanoemulsion (Nano+VitD) or 1.8 mL of canola oil followed by 1.2 mL delivering the same dose of VitD3 obtained from a commonly-prescribed commercially available product (70 µg/mL VitD; VIDROP, MUP Co., Egypt) (Oil+VitD). Their control groups received the same volumes of delivery vehicles, Nano-VitD or Oil-VitD, respectively. All treated groups received their doses every other day (3 doses total) within 1 week by gavage. Figure 1 shows the experimental design, the subgroups and amounts of VitD provided.

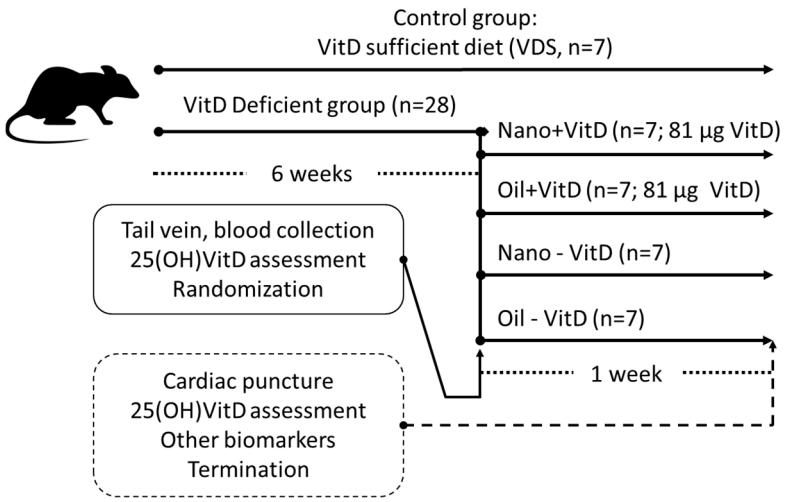

Figure 1. Study experimental design.

2.5 Blood Sampling and biochemical analysis

Blood samples were collected from the lateral tail vein after six weeks of dietary conditioning. Serum samples were obtained after blood clothing at room temperature for one hour and centrifugation at 2,000 x g for 10 minutes at 4°C. This blood sample was used to determine 25(OH)VitD in serum. After one week of treatment, all animals were terminated by first using isoflurane, followed by cardiac puncture and cervical dislocation. Blood collected at this point was used for the determination of 25(OH)VitD, parathyroid hormone (PTH), Ca, P and alkaline phosphatase activity (ALP). Right and left femur bone was used to prepare histopathological sections. Plasma was separated by centrifugation and stored at -80 °C until analysis.

Serum 25(OH)VitD and PTH assays. 25(OH)VitD and PTH concentrations in serum were determined using ELISA kits (MYBio-Source, San Diego, CA, USA; #MBS2601819 & #MBS265580, respectively). Serum 25(OH)VitD and PTH for all specimens and controls were conducted concurrently with the standards, and concentrations were calculated from external standards. The coefficient of variance of the 25(OH)VitD and PTH were 10 and 20%, respectively.

Ca, P, and ALP. Ca (#MBS8243246) and P (# MBS8243207) concentration and alkaline phosphatase activity (ALP) (# MBS2540468) in serum were analyzed using colorimetric kits following the steps of the manufacturer's instructions provided along with the kits (MYBio-Source, San Diego, CA, USA). The inter-day coefficient of variation for each of the assays was 10, 10, and 20% for Ca, P, and ALP, respectively.

2.6 Histological and Histomorphometric Analysis

The femur specimens from all rats were collected. Femur samples of VDS group were labeled as 'controls'. All specimens were decalcified, sectioned, processed and stained by Hematoxylin, and Eosin. Gross and microscopic description were done for all femur samples and they were rated according to the criteria as mild, moderate or severe osteoporotic changes [30]. The histomorphometric analysis of the selected regions of interest (ROIs) was done by Image J software (Image J, National Institutes of Health, USA). Tools of Image J were used to outline and calculate the osteoid area and the area of bone marrow separating the bone trabeculae (trabecular separation) [31]. Two ROIs from each rat sample were selected for analysis and the presented as mean±SD.

2.7 Statistical Analysis

Data were expressed as mean ± SD. Before and after effects of dietary treatments on 25(OH)VitD was evaluated using paired t-test. ANOVA with Tukey HSD post hoc test were used to compare group effects on all variables. All differences were considered significant at an alpha of 0.05. All statistical analyses were conducted using the SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Changes of 25(OH)VitD and biomarkers of VitD deficiency effects on bone

Table 1 shows levels of 25(OH)VitD in serum for all study groups before and after one-week of treatment. After one week of treatment, VitD deficient animals receiving VitD dispersed in the PPI nanoemulsion showed a higher circulating level of VitD (34.38±7.00 vs. 14.65±1.29 nmol/L, $p<0.001$). Rats receiving VitD mixed in canola oil showed no improvement in this parameter ($p>0.05$). There were no differences in animal body weight after seven weeks of experiments (Table 2). Provision of VitD stably dispersed in PPI nanoemulsion restored the levels of biomarkers of VitD deficiency as compared to the positive control, the other delivery vehicle controls, and the Oil+VitD group (Table 2).

3.2 Histological changes after treament with VitD deficient diets and therapies

Gross examination of cut sections after six-weeks of VitD-deficient diet revealed no significant histopathological changes. However, as shown in Figure 2, microscopically regions of interest (ROIs) of examined sections revealed rat bone tissue showing reduced thickening of the bone cortex (osteoid area) associated with widely separated bone trabeculae (trabecular separation) containing bone marrow element.

Table 1. Levels of 25(OH)VitD in serum before and after one week of treatment.

	25(OH)VitD (nmol/L)[2]		
Groups[1]	*Before*	*After*	*P-value*
VDS	31.68 ± 10.40	36.84 ± 9.16	0.352
Nano - VitD	15.38 ± 5.51	15.88 ± 5.77	0.363
Oil - VitD	18.26 ± 6.38	15.59 ± 2.45	0.421
Nano+VitD	14.65 ± 1.29	34.37 ± 7.00	<0.001
Oil+VitD	14.33 ± 3.43	14.05 ± 3.08	0.356

[1] Animal groups receiving: VitD sufficient diet (VDS), VitD dispersed in nanoemulsion (Nano+VitD), VitD mixed in oil (Oil+VitD), nanoemulsion without VitD (Nano - VitD), and canola oil without VitD (Oil - VitD).

[2] Results are presented as Means ± SD. Paired t-test was used to test before and after effects ($p<0.05$).

Table 2. Concentration of several blood biomarkers of VitD deficiency in rats after one week of dietary treatments.

Groups[1]	*Wt (g)*	*25(OH)VitD (nmol/L)*[2]	*PTH (pg/mL)*	*Ca (mg/dL)*	*P (mg/dL)*	*ALP (U/L)*
VDS	262.28 ± 36.98	36.84 ± 9.16 [a]	23.36 ± 12.00 [a]	10.24 ± 0.92 [a]	3.67 ± 1.13 [a]	58.5 ± 11.5 [a]
Nano - VitD	270.45 ± 31.72	15.88 ± 5.77 [b]	37.54 ± 6.61 [a]	7.12 ± 1.16 [b]	1.38 ± 0.57 [b]	196.2 ± 57.7 [b]
Oil - VitD	249.48 ± 26.96	15.59 ± 2.45 [b]	78.93 ± 8.31 [b]	6.68 ± 1.92 [b]	1.17 ± 0.62 [b]	171.0 ± 17.6 [b]
Nano+VitD	253.11 ± 26.64	34.37 ± 7.00 [a]	25.22 ± 14.26 [a]	9.64 ± 0.60 [a]	3.65 ± 0.71 [a]	72.4 ± 31.0 [a]
Oil+VitD	266.13 ± 27.53	14.05 ± 3.08 [b]	86.05 ± 9.67 [b]	5.32 ± 1.28 [b]	1.33 ± 0.32 [b]	182.6 ± 61.8 [b]

[1] Animal groups receiving: VitD sufficient diet (VDS), VitD dispersed in nanoemulsion (Nano+VitD), VitD mixed in oil (Oil+VitD), nanoemulsion without VitD (Nano - VitD), and canola oil without VitD (Oil - VitD).

[2] Results are presented as Means ± SD. Different superscripts within each column represent statistical differences after One-way ANOVA and Tukey's HSD test ($p<0.05$).

After one week of dietary treatment, a significant improvement of the osteoid area rather than trabecular separation of the bone sections was observed by histomorphometric parameters of Image J software in the Nano+VitD group its deficient counterpart as shown Figure 3. As can be seen in Figure 4, however, consumption of VitD in canola oil failed to produce a significant improvement in the osteoid area compared with its counterpart. An increase in trabecular separation was observed in animals receiving canola oil with VitD.

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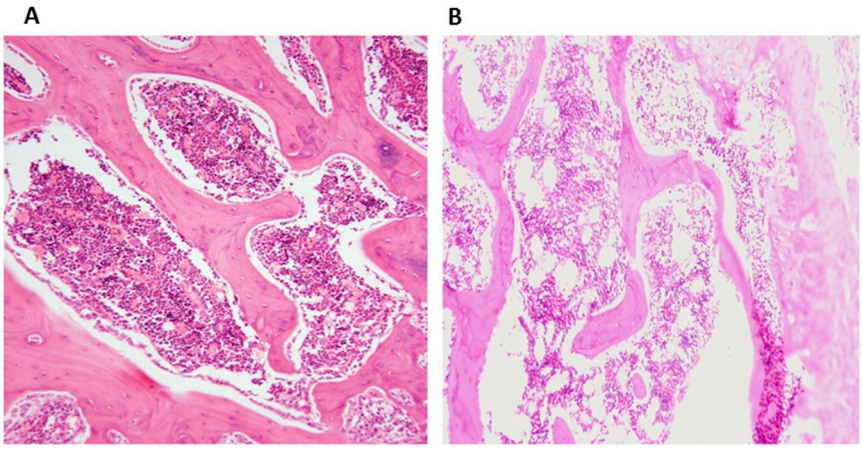

Figure 2. Comparison of histological sections of rat's femur from VitD-sufficient group (A) or -deficient group (B).

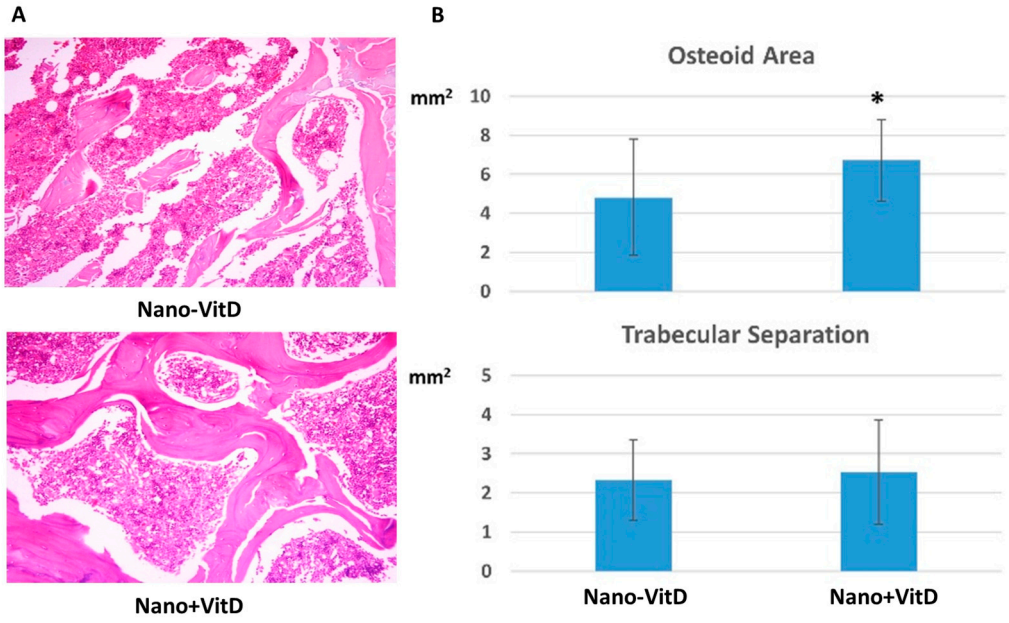

Figure 3. (A) Histopathological changes in femur of rats receiving Nano+VitD vs. Nano-VitD after one week of dietary treatment. (B) Histomorphometric parameters osteoid area and trabecular separation. *Indicates significant differences after t-test ($p<0.05$).

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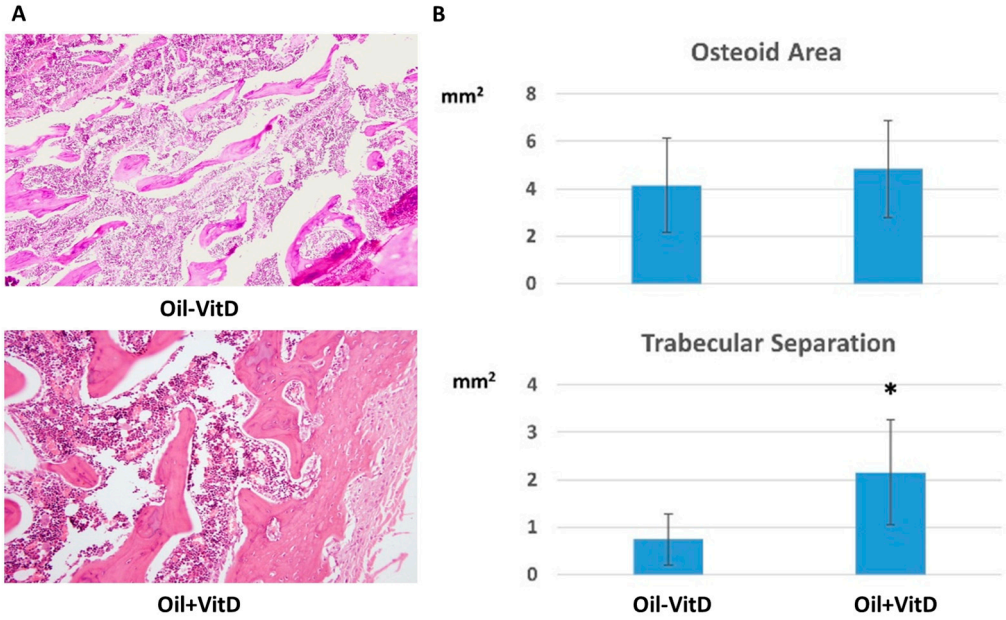

Figure 4. (A) Histopathological changes in femur of rats receiving Oil+VitD vs. Oil-VitD after one week of dietary treatment. (B) Histomorphometric parameters osteoid area and trabecular separation. *Indicates significant differences after t-test ($p<0.05$).

4. Discussion

In the present study, we show that consumption of VitD stably dispersed in a PPI nanoemulsion, created by sonication and pH shifting combined treatment as shown in our previous work [27] resulted in improved VitD status and a near complete recovery from the symptoms of vitamin D deficiency (VDD). After one week of treatment, only the group receiving VitD (as cholecalciferol) dispersed in PPI nanoemulsions showed improvement in 25(OH)VitD, the circulating form of VitD and biomarker of its status [1]. Moreover, animals receiving the Nano+VitD treatment showed an expected directional change in all blood biomarkers of bone turnover indicative of recovery from deficiency (e.g., lower PTH and ALP, and higher Ca and P) closer to the levels found in the VitD-sufficient control. The increase in serum 25(OH)VitD was 2.3 times higher than its control (Nano - VitD) after one-week of treatment, potentially due to increased protection of the vitamin during digestion [27] and enhancement of oral bioavailability due to improved micellarization of VitD in the small intestine [32,33]. The group receiving Oil+VitD did not improve 25(OH)VitD status. This could be due to lower absorption. The Vidrop product disperses cholecalciferol in β-cyclodrextrin and polysorbate 20. According to the vendor's recommendations for treatment of florid rickets, the product should be consumed daily for at least 3 weeks. In a recent study, Kadappan et al., (2018) created vitamin D-containing nanoemulsions using Q-NATURALE® 200V, an extract from *Quillaia Saponaria* Molina, as surfactant and showed enhanced micellarization of VitD and absorption in rats [33]. Moreover, after single dose administration of nanoemulsified lipophilic vitamin mixture (i.e., vitamins A, D, E) to rats showed higher plasma area under the curve concentration for these vitamins compared to controls [34]. Though these studies inform on the bioavailability of vitamin D in nanoemulsions, it is important to consider its functionality and efficacy.

Nutrient depletion/repletion models in animals are useful to study the functional impact of new delivery vehicles that can be added into the food supply. We used the model of Fleet et al., (2008) to promote VDD in rats. Though there were not changes in body weight, serum 25(OH)VitD levels were markedly different between the sufficient and deficient groups before randomization. Vitamin deficiency was ascertained by attaining low levels of 25(OH)VitD as well as changes in bone turnover biomarkers such as increased PTH signaling (secondary hyperparathyroidism) and alkaline phosphatase activity in blood. The expected trend changes due to VDD were similar, though not to the same extent, to those found in previous studies with VDD in rats [35]. PTH is an important

hormone for bone metabolism, maintaining the normal serum concentrations of calcium and phosphorous. Increased PTH concentrations will lead to an increase in bone turnover, causing negative bone balance and an increased fracture risk [36]. Deficiency of VitD results in secondary hyperparathyroidism, a symptom common in rickets and osteomalacia [37]. Moreover, ALP plays a role in bone mineralization and phosphorus homeostasis [38]. It is has been shown to increase during osteomalacia and rickets [39,40], however it is not a reliable biomarker of vitamin D deficiency [41].

VitD is important in regulating homeostasis of Ca and P, and severe VDD is known to increase the risk of bone and kidneys [42]. The active or hormonal form of VitD [1,25$(OH)_2$VitD] binds to the VitD receptor in the nucleus and triggers a series of effects in several tissues including the kidney, the bone, and the intestines, all which increase serum Ca [43,44]. Although VitD interacts with PTH [43], hypovitaminosis D does not influence PTH under sufficient serum levels of Ca, in the kidneys, VitD is regulated by PTH, Ca and P levels [45]. Moreover, hypophosphatemia was observed in all groups except the VDS as well as the Nano+VitD groups. It is well known that a certain degree of phosphate depletion may develop in VDD [46]. Renal transport of Ca is known to be affected by phosphate transport. It has been reported that the depletion of phosphate is related to hypercalciuria [47,48] with a resistance to PTH actions to stimulate tubular reabsorption of Ca [47], to inhibit tubular reabsorption of phosphate [49], and consequently leads to raise serum Ca in blood. The occurrence of hypercalciuria by phosphate depletion has been attributed to the increase in the filtered load of Ca and combined with elevation of ALP concentrations in VDD groups. The Nano+VitD group showed normal VitD levels, which may be due to accompanying improvement of calcium level, and the decrease in the tubular reabsorption due to secondary amelioration of the functional hypoparathyroidism [50,51]. Also, Coburn and Massry, (1970) have suggested that hypophosphatemia can alter renal handling of Ca and reduces the responsiveness to PTH [47]. High levels of PTH were remarked with adverse effects on bone resorption and low bone mass [52]. The mature osteoclast removes calcium and phosphorus from the bone to maintain blood calcium and phosphorus levels. Adequate calcium and phosphorus levels promote the mineralization of the skeleton [1].

Nanoemulsions are an alternative option to deliver lipid-soluble nutrients and bioactive compounds [53]. Nanoemulsions are colloidal dispersions that contain small particles (20-200 nm) amenable for the dispersion of lipophilic substances such fat-soluble vitamins dispersed within an aqueous medium and known to increase their oral bioavailability [32,54,55]. This study is consistent with those findings of others showing that reduction of particle size of liposoluble bioactives and their stabilization using different surfactants results in their increased absorption [32,54,55]. For instance, nanoemulsification has increased the oral bioavailability in rats of anti-inflammatory drugs emulsified with Tween 20 [56] or anti-cancer bioactives emulsified with Cremorphor EL and polyethylene glycol [57], bioactives such as Kenaf seed oil emulsified with sodium caseinate and Tween 20 [58], vitamin E emulsified in lecithin [59], and coenzyme Q10 emulsified with salmon lecithin [60]. In the case of food applications and due to public pressure for clean labels, the food industry avidly seeks food-grade, natural surfactants, which are usually made of proteins, polysaccharides, or their combination [61,62]. In this study, stabilized PPI was used as a suitable protein-based surfactant with the ability to disperse and protect VitD [27,63]. PPI is an intriguing surfactant as it is plant-based, hypoallergenic, possesses high hydrophobicity, and is commercially available [10,64].

Although to a great extent eradicated from many countries, VDD remains a public health concern all over the world [6,65,66]. Prevalence rates of VDD and insufficiency are high among all age groups, where women and children are most vulnerable groups [65–69]. Infants are at a higher risk of VDD or VitD insufficiency if they are born from mothers who are of young age, who had multiple pregnancies, and are non-White race/ethnicity [70]. Non-White populations (skin type V) are at a higher risk of VDD and VitD insufficiency because they are unable to efficiently synthesize VitD in the skin [71,72]. In Middle Eastern countries, where excessive heat, life style choices, and cultural norms reduces sunlight exposure of populations groups, VDD and insufficiency are highly prevalent [73]. Moreover, VitD is difficult to obtain from the ordinary diet because it is not naturally

present in many foods. Thus, food fortification with VitD has been proposed as a nutrition specific strategy with the widest reach and impact in the population in terms of enhancing VitD status [7].

Current fortification technologies are limited to a few target foods (e.g., oil and dairy products). This is due to the high sensitivity of VitD to oxidation, UV light, and temperature as well as the potential deleterious effects of new technologies on food attributes (e.g., color, flavor), which could affect consumer compliance [74]. Despite the existence of, albeit limited, fortified products, fortification strategies have had limited impact in several countries due to regulatory, monitoring and evaluation issues [75], limited number of suitable foods for VitD fortification [76], and most importantly, lack of culturally appropriate fortification delivery systems. Our study addresses these identified problems by proposing the use of nanoemulsions to disperse and protect VitD within foods as well as enhancing its absorption. The results of the present study will help in devising a strategy that may increase VitD efficacy as a fortificant and hence assist in global efforts to reduce VDD.

5. Conclusions

The evidence from this study suggests that the consumption of VitD dispersed in PPI nanoemulsion created by ultrasound and pH shifting enhances its absorption and restores its status and biomarkers of bone turnover in VitD-deficient rats. This is potentially due to a faster absorption due to improved micellarization. Future studies should focus on evaluating the potential toxicity of VitD delivered in PPI nanoemulsions as well as determining their potential effects on foods.

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References

1. Holick, M. F. The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention. *Rev. Endocr. Metab. Disord.* 2017, *18*, 153–165.
2. Iom, T.; Intakes, D. R. Institute of Medicine (IOM). Dietary reference intakes for calcium and vitamin D. Washington DC: The National Academies Press; 2011. *Pediatrics* **2012**, *130*, e1424.
3. Alshahrani, F.; Aljohani, N. Vitamin D: Deficiency, sufficiency and toxicity. *Nutrients* **2013**, *5*, 3605–3616.
4. McKenna, M. J.; Murray, B. Vitamin D deficiency. In *Endocrinology and Diabetes: A Problem-Oriented Approach*; 2014; Vol. 9781461486, pp. 293–304.
5. Wang, H.; Chen, W.; Li, D.; Yin, X.; Zhang, X.; Olsen, N.; Zheng, S. G. Vitamin D and Chronic Diseases. *Aging Dis.* **2017**, *8*, 346–353.
6. Roth, D. E.; Abrams, S. A.; Aloia, J.; Bergeron, G.; Bourassa, M. W.; Brown, K. H.; Calvo, M. S.; Cashman, K. D.; Combs, G.; De-Regil, L. M.; Jefferds, M. E.; Jones, K. S.; Kapner, H.; Martineau, A. R.; Neufeld, L. M.; Schleicher, R. L.; Thacher, T. D.; Whiting, S. J. Global prevalence and disease burden of vitamin D deficiency: a roadmap for action in low- and middle-income countries. *Ann. N. Y. Acad. Sci.* **2018**, *1430*, 44–79.
7. Cashman, K. D.; Kiely, M. Vitamin D and Food Fortification. In *Vitamin D*; 2018; pp. 109–127.
8. Park, S. J.; Garcia, C. V.; Shin, G. H.; Kim, J. T. Development of nanostructured lipid carriers for the encapsulation and controlled release of vitamin D3. *Food Chem.* **2017**, *225*, 213–219.
9. Shu, G.; Khalid, N.; Zhao, Y.; Neves, M. A.; Kobayashi, I.; Nakajima, M. Formulation and stability assessment of ergocalciferol loaded oil-in-water nanoemulsions: Insights of emulsifiers effect on stabilization mechanism. *Food Res. Int.* **2016**, *90*, 320–327.
10. Ozturk, B.; Argin, S.; Ozilgen, M.; McClements, D. J. Nanoemulsion delivery systems for oil-soluble vitamins: Influence of carrier oil type on lipid digestion and vitamin D3 bioaccessibility. *Food Chem.* **2015**, *187*, 499–506.
11. Mohammadi, M.; Ghanbarzadeh, B.; Hamishehkar, H. Formulation of nanoliposomal vitamin D3 for potential application in beverage fortification. *Adv. Pharm. Bull.* **2014**, *4*, 569–575.
12. Diarrassouba, F.; Garrait, G.; Remondetto, G.; Alvarez, P.; Beyssac, E.; Subirade, M. Food protein-based microspheres for increased uptake of Vitamin D3. *Food Chem.* **2015**, *173*, 1066–1072.
13. Hasanvand, E.; Fathi, M.; Bassiri, A. Production and characterization of vitamin D3-loaded starch nanoparticles: effect of amylose to amylopectin ratio and sonication parameters. *J. Food Sci. Technol.* **2018**, *Volume 55*, 1314–1324.
14. Teng, Z.; Luo, Y.; Wang, Q. Carboxymethyl chitosan-soy protein complex nanoparticles for the encapsulation and controlled release of vitamin D3. *Food Chem.* **2013**, *141*, 524–532.
15. Sharma, A.; Sharma, U. S. Liposomes in drug delivery: Progress and limitations. *Int. J. Pharm.* 1997, *154*, 123–140.
16. McClements, D. J. Encapsulation, protection, and release of hydrophilic active components: Potential and limitations of colloidal delivery systems. *Adv. Colloid Interface Sci.* **2015**, *219*, 27–53.
17. Jain, K.; Kumar Mehra, N.; Jain, N. K. Nanotechnology in drug delivery: Safety and toxicity issues. *Curr. Pharm. Des.* **2015**, *21*, 4252–4261.
18. Wang, Q.; Allen, J. C.; Swaisgood, H. E. Binding of Vitamin D and Cholesterol to β-Lactoglobulin. *J. Dairy Sci.* **1997**, *80*, 1054–1059.
19. Yang, M. C.; Guan, H. H.; Liu, M. Y.; Lin, Y. H.; Yang, J. M.; Chen, W. L.; Chen, C. J.; Mao, S. J. T. Crystal structure of a secondary vitamin D3-binding site of milk β-lactoglobulin. *Proteins Struct. Funct. Genet.* **2008**, *71*, 1197–1210.

20. Yang, M. C.; Guan, H. H.; Yang, J. M.; Ko, C. N.; Liu, M. Y.; Lin, Y. H.; Huang, Y. C.; Chen, C. J.; Mao, S. J. T. Rational design for crystallization of β-lactoglobulin and vitamin D3complex: Revealing a secondary binding site. In *Crystal Growth and Design*; 2008; Vol. 8, pp. 4268–4276.

21. Kohl, E. A.; Schaefer, P. C. Improved high-pressure liquid chromatographic assay of serum 25-hydroxycholecalciferol and 25-hydroxyergocalctferol after reverse-phase sep-pak C18cartridge preparation of sample. *J. Liq. Chromatogr.* **1981**, *4*, 2023–2037.

22. Kao, P. C.; Heser, D. W. Simultaneous determination of 25-hydroxy- and 1,25-dihydroxyvitamin D from a single sample by dual-cartridge extraction. *Clin. Chem.* **1984**, *30*, 56–61.

23. Foegeding, E. A.; Davis, J. P. Food protein functionality: A comprehensive approach. *Food Hydrocoll.* **2011**, *25*, 1853–1864.

24. Lam, R. S. H.; Nickerson, M. T. Food proteins: A review on their emulsifying properties using a structure-function approach. *Food Chem.* 2013, *141*, 975–984.

25. Beverung, C. J.; Radke, C. J.; Blanch, H. W. Protein adsorption at the oil/water interface: Characterization of adsorption kinetics by dynamic interfacial tension measurements. *Biophys. Chem.* **1999**, *81*, 59–80.

26. Karaca, A. C.; Low, N.; Nickerson, M. Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Res. Int.* **2011**, *44*, 2742–2750.

27. Jiang, S.; Ding, J.; Andrade, J.; Rababah, T. M.; Almajwal, A.; Abulmeaty, M. M.; Feng, H. Modifying the physicochemical properties of pea protein by pH-shifting and ultrasound combined treatments. *Ultrason. Sonochem.* **2017**, *38*, 835–842.

28. Reeves, P. G.; Nielsen, F. H.; Fahey, G. C. AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. *J. Nutr.* **1993**, *123*, 1939–1951.

29. Fleet, J. C.; Gliniak, C.; Zhang, Z.; Xue, Y.; Smith, K. B. Serum metabolite profiles and target tissue gene expression define the effect of cholecalciferol intake on calcium metabolism in rats and mice. *J. Nutr.* **2008**, *138*, 1114–1120.

30. Itakura, C.; Yamasaki, K.; Goto, M. Pathology of experimental vitamin D deficiency rickets in growing chickens. II. Parathyroid gland. *Avian Pathol.* **1978**, *7*, 515–532.

31. Egan, K. P.; Brennan, T. A.; Pignolo, R. J. Bone histomorphometry using free and commonly available software. *Histopathology* **2012**, *61*, 1168–1173.

32. Salvia-Trujillo, L.; Martín-Belloso, O.; McClements, D. Excipient Nanoemulsions for Improving Oral Bioavailability of Bioactives. *Nanomaterials* **2016**, *6*, 17.

33. Kadappan, A. S.; Guo, C.; Gumus, C. E.; Bessey, A.; Wood, R. J.; McClements, D. J.; Liu, Z. The Efficacy of Nanoemulsion-Based Delivery to Improve Vitamin D Absorption: Comparison of In Vitro and In Vivo Studies. *Mol. Nutr. Food Res.* **2018**, *62*, 1–8.

34. Lee, Y.-J.; Kwon, M.; Kim, T.-H.; Kim, K.; Jeong, S.-H.; Chang, H.-R. Pharmacokinetic Characterization of Nano-emulsion Vitamin A, D and E (LaVita) in Rats. *Korean J. Environ. Agric.* **2011**, *30*, 196–201.

35. Toromanoff, A.; Ammann, P.; Mosekilde, L.; Thomsen, J. S.; Riond, J. L. Parathyroid hormone increases bone formation and improves mineral balance in vitamin D-deficient female rats. *Endocrinology* **1997**, *138*, 2449–2457.

36. Khundmiri, S. J.; Murray, R. D.; Lederer, E. PTH and Vitamin D. *Compr. Physiol.* **2016**, *6*, 561–601.

37. Lips, P.; Van Schoor, N. M. The effect of vitamin D on bone and osteoporosis. *Best Pract. Res. Clin. Endocrinol. Metab.* **2011**, *25*, 585–591.

38. Millán, J. L. The role of phosphatases in the initiation of skeletal mineralization. *Calcif. Tissue Int.* **2013**, *93*,

299–306.

39. Chuang, L. H.; Tung, Y. C.; Liu, S. Y.; Lee, C. T.; Chen, H. L.; Tsai, W. Y. Nutritional rickets in Taiwanese children: Experiences at a single center. *J. Formos. Med. Assoc.* **2018**, *117*, 583–587.
40. Bhambri, R.; Naik, V.; Malhotra, N.; Taneja, S.; Rastogi, S.; Ravishanker, U.; Mithal, A. Changes in Bone Mineral Density Following Treatment of Osteomalacia. *J. Clin. Densitom.* **2006**, *9*, 120–127.
41. Shaheen, S.; Noor, S. S.; Barakzai, Q. Serum alkaline phosphatase screening for vitamin D deficiency states. *J. Coll. Physicians Surg. Pakistan* **2012**, *22*, 424–427.
42. Uchida, H.; Kurata, Y.; Hiratsuka, H.; Umemura, T. The effects of a vitamin D - Deficient diet on chronic cadmium exposure in rats. *Toxicol. Pathol.* **2010**, *38*, 730–737.
43. DeLuca, H. F. Overview of general physiologic features and functions of vitamin D. *Am. J. Clin. Nutr.* **2004**, *80*, 1689S–1696S.
44. Lips, P. Vitamin D physiology. *Prog. Biophys. Mol. Biol.* **2006**, *92*, 4–8.
45. Need, A. G.; O'Loughlin, P. D.; Morris, H. A.; Coates, P. S.; Horowitz, M.; Nordin, B. E. C. Vitamin D metabolites and calcium absorption in severe vitamin D deficiency. *J. Bone Miner. Res.* **2008**, *23*, 1859–1863.
46. Brautbar, N.; Walling, M. W.; Coburn, J. W. Interactions between vitamin D deficiency and phosphorus depletion in the rat. *J. Clin. Invest.* **1979**, *63*, 335–341.
47. Coburn, J. W.; Massry, S. G. Changes in serum and urinary calcium during phosphate depletion: studies on mechanisms. *J. Clin. Invest.* **1970**, *49*, 1073–1087.
48. Clark, I.; Rivera-Cordero, F. Effects of endogenous parathyroid hormone on calcium, magnesium and phosphate metabolism in rats. II. Alterations in dietary phosphate. *Endocrinology* **1974**, *95*, 360–369.
49. Steele, T. H. Renal resistance to parathyroid hormone during phosphorus deprivation. *J. Clin. Invest.* **1976**, *58*, 1461–1464.
50. Coburn, K. R. Preliminary investigation of bone change as a result of exposure to reduced atmospheric pressure. *Aerosp. Med.* **1970**, *41*, 188–190.
51. Rader, J. I.; Howard, G. A.; Feist, E.; Turner, R. T.; Baylink, D. J. Bone mineralization and metabolism of3H-25-hydroxyvitamin D3 in thyroparathyroidectomized rats treated with parathyroid extract. *Calcif. Tissue Int.* **1979**, *29*, 21–26.
52. Khaw, K.; Sneyd, M.; Compston, J. Bone density, parathyroid hormone and 25-hydroxyvitaminD concentrations in middle-aged women. *Br. Med. J.* **1992**, *305*, 273–277.
53. Bonifácio, B. V.; da Silva, P. B.; Aparecido dos Santos Ramos, M.; Maria Silveira Negri, K.; Maria Bauab, T.; Chorilli, M. Nanotechnology-based drug delivery systems and herbal medicines: A review. *Int. J. Nanomedicine* **2013**, *9*, 1–15.
54. Silva, A. C.; Santos, D.; Ferreira, D.; Lopes, C. M. Lipid-Based Nanocarriers as an Alternative for Oral Delivery of Poorly Water-Soluble Drugs: Peroral and Mucosal Routes. *Curr. Med. Chem.* **2012**, 4495–4510.
55. McClements, D. J. Edible lipid nanoparticles: Digestion, absorption, and potential toxicity. *Prog. Lipid Res.* 2013, *52*, 409–423.
56. Yen, C. C.; Chen, Y. C.; Wu, M. T.; Wang, C. C.; Wu, Y. T. Nanoemulsion as a strategy for improving the oral bioavailability and anti-inflammatory activity of andrographolide. *Int. J. Nanomedicine* **2018**, *13*, 669–680.
57. Sun, L.; Wan, K.; Hu, X.; Zhang, Y.; Yan, Z.; Feng, J.; Zhang, J. Functional nanoemulsion-hybrid lipid nanocarriers enhance the bioavailability and anti-cancer activity of lipophilic diferuloylmethane. *Nanotechnology* **2016**, *27*, 085102.
58. Cheong, A. M.; Tan, C. P.; Nyam, K. L. Effect of Emulsification Method and Particle Size on the Rate of in vivo Oral Bioavailability of Kenaf (Hibiscus cannabinus L.) Seed Oil. *J. Food Sci.* **2018**, *83*, 1964–1969.

59. Saratale, R. G.; Lee, H. S.; Koo, Y. E.; Saratale, G. D.; Kim, Y. J.; Imm, J. Y.; Park, Y. Absorption kinetics of vitamin E nanoemulsion and green tea microstructures by intestinal in situ single perfusion technique in rats. *Food Res. Int.* **2018**, *106*, 149–155.
60. Belhaj, N.; Dupuis, F.; Arab-Tehrany, E.; Denis, F. M.; Paris, C.; Lartaud, I.; Linder, M. Formulation, characterization and pharmacokinetic studies of coenzyme Q10PUFA's nanoemulsions. *Eur. J. Pharm. Sci.* **2012**, *47*, 305–312.
61. Kralova, I.; Sjöblom, J. Surfactants used in food industry: A review. *J. Dispers. Sci. Technol.* **2009**, *30*, 1363–1383.
62. McClements, D. J.; Rao, J. Food-Grade nanoemulsions: Formulation, fabrication, properties, performance, Biological fate, and Potential Toxicity. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 285–330.
63. Yerramilli, M.; Longmore, N.; Ghosh, S. Stability and Bioavailability of Curcumin in Mixed Sodium Caseinate and Pea Protein Isolate Nanoemulsions. *JAOCS, J. Am. Oil Chem. Soc.* **2018**, *95*, 1013–1026.
64. Malekzad, H.; Mirshekari, H.; Sahandi Zangabad, P.; Moosavi Basri, S. M.; Baniasadi, F.; Sharifi Aghdam, M.; Karimi, M.; Hamblin, M. R. Plant protein-based hydrophobic fine and ultrafine carrier particles in drug delivery systems. *Crit. Rev. Biotechnol.* **2018**, *38*, 47–67.
65. Mithal, A.; Wahl, D. A.; Bonjour, J. P.; Burckhardt, P.; Dawson-Hughes, B.; Eisman, J. A.; El-Hajj Fuleihan, G.; Josse, R. G.; Lips, P.; Morales-Torres, J. Global vitamin D status and determinants of hypovitaminosis D. *Osteoporos. Int.* **2009**, *20*, 1807–1820.
66. Holick, M. F. High prevalence of vitamin D inadequacy and implications for health. *Mayo Clin. Proc.* **2006**, *81*, 353–373.
67. Glerup, H. [Vitamin D deficiency among immigrants]. *Ugeskr Laeger* **2000**, *162*, 6196–6199.
68. Kauppinen-Mäkelin, R.; Tähtelä, R.; Löyttyniemi, E.; Kärkkäinen, J.; Välimäki, M. J. A high prevalence of hypovitaminosis D in Finnish medical in- and outpatients. *J. Intern. Med.* **2001**, *249*, 559–563.
69. Thomas, M. K.; Lloyd-Jones, D. M.; Thadhani, R. I.; Shaw, A. C.; Deraska, D. J.; Kitch, B. T.; Vamvakas, E. C.; Dick, I. M.; Prince, R. L.; Finkelstein, J. S. Hypovitaminosis D in Medical Inpatients. *N. Engl. J. Med.* **1998**, *338*, 777–783.
70. Marshall, I.; Mehta, R.; Ayers, C.; Dhumal, S.; Petrova, A. Prevalence and risk factors for vitamin D insufficiency and deficiency at birth and associated outcome. *BMC Pediatr.* **2016**.
71. Kift, R.; Berry, J. L.; Vail, A.; Durkin, M. T.; Rhodes, L. E.; Webb, A. R. Lifestyle factors including less cutaneous sun exposure contribute to starkly lower vitamin D levels in U.K. South Asians compared with the white population. *Br. J. Dermatol.* **2013**, *169*, 1272–1278.
72. Clemens, T. L.; Henderson, S. L.; Adams, J. S.; Holick, M. F. Increased Skin Pigment reduces the capacity of skin to synthesise vitamin D3. *Lancet* **1982**, *1*, 74–76.
73. Al Jurayyan, N. A.; Mohamed, S.; A Al Issa, S. D.; Al Jurayyan, A. N. Rickets and osteomalacia in Saudi children and adolescents attending endocrine clinic, Riyadh, Saudi Arabia. *Sudan. J. Paediatr. Sudan J Paediatr Sudan. J. Paediatr.* **2012**, *1212*, 56–63.
74. Gonnet, M.; Lethuaut, L.; Boury, F. New trends in encapsulation of liposoluble vitamins. *J. Control. Release* **2010**, *146*, 276–290.
75. Holick, M. F. Calcium and vitamin D. Diagnostics and therapeutics. *Clin Lab Med* **2000**, *20*, 569–590.
76. Murphy, S. C.; Whited, L. J.; Rosenberry, L. C.; Hammond, B. H.; Bandler, D. K.; Boor, K. J. Fluid milk vitamin fortification compliance in New York State. *J. Dairy Sci.* **2001**, *84*, 2813–2820.