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Food fortification and biofortification as potential strategies for prevention of vitamin D deficiency

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Running title: 25-hydroxyvitamin D\textsubscript{3} fortified foods

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Abstract

Hypovitaminosis D is widespread throughout the world. The cutaneous production of vitamin D through sunlight can be limited by several factors (e.g. skin pigmentation, sunscreen usage and, increasingly, indoor lifestyle). Thus, diet has become an important strategy to increase vitamin D intake and status. However, there are a limited number of foods (e.g. eggs, oily fish and wild mushroom) naturally enriched with vitamin D, and concentrations can vary significantly between and within species. Therefore, the need for vitamin D fortified foods (including via direct fortification and biofortification) to support adequacy of vitamin D status [blood 25-hydroxivitamin D (25(OH) D)] is a corollary of several limitations to synthesise vitamin D from sunlight. Ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) can be found in some mushrooms and animal-derived foods, respectively. Evidence has shown vitamin D₃ is more effective than vitamin D₂ at raising 25(OH) D blood concentrations. The vitamin D metabolite, 25(OH) D₃, is present in animal-derived foods (e.g. meat, eggs and fish), and several intervention trials have shown 25(OH) D₃ to be more effective at raising blood 25(OH) D concentrations than vitamin D₃. In addition, 25(OH) D₃ supplements may prove to be preferable to vitamin D₃ for patients with certain clinical conditions. However, there is limited evidence on the effect of 25(OH) D₃ fortified foods on human vitamin D status and health. Therefore, long-term randomised controlled trials to evaluate the effect of 25(OH) D₃ fortified foods on vitamin D status are needed for both the general population and patients with certain conditions.

Key words

Vitamin D, 25(OH) D, fortification, biofortification, randomised controlled trial, dairy
Introduction

Vitamin D is a lipid soluble vitamin that acts as a hormone (Nair & Maseeh 2012), which generally refers to ergocalciferol (vitamin D$_2$) and cholecalciferol (vitamin D$_3$) (Tripkovic et al. 2012). Vitamin D$_2$ and vitamin D$_3$ are produced by fungi and the skin of vertebrates, respectively (Wacker & Holick 2013). The role of vitamin D in musculoskeletal health is well established (Wolff et al. 2008). Recently, vitamin D deficiency has been suggested to be associated with several non-musculoskeletal health outcomes, such as cardiovascular disease, certain cancers and type 2 diabetes, although mechanisms are not clear (Wang et al. 2017).

Vitamin D status is assessed by measuring the blood concentration of circulating 25-hydroxyvitamin D (25(OH) D) (Holick 2009). Widespread hypovitaminosis D is now acknowledged (Hilger et al. 2014), although there is some dispute about the thresholds for vitamin D deficiency and insufficiency (Spiro & Buttriss 2014). In the UK, vitamin D deficiency is defined as 25(OH) D $<$25 nmol/L (SACN 2016). The UK National Diet and Nutritional Survey (NDNS) reported that in 2008-2012 24% men and 21.7% of women (aged 19-64 years) had vitamin D deficiency (Bates et al. 2014). With seasonal variation, the prevalence of hypovitaminosis D in the UK was alarmingly high during winter and spring. A cross-sectional study conducted in the UK by Hypponen and Power (2007) reported that during the winter and spring months 25(OH) D concentrations were $<$25 nmol/L, $<$40 nmol/L and $<$75 nmol/L in 15.5%, 46.6% and 87.1% of participants, respectively. There are several additional contributors to hypovitaminosis D, such as skin pigmentation, sunscreen usage, and an increasingly indoor lifestyle, all of which reduce the cutaneous production of vitamin D (Holick 2004). Furthermore, vitamin D supplement can also contributes to vitamin D intake, however, uptake of supplements tends to be low (Hennessy et al. 2017; Datta et al. 2016 ). As a result, dietary intake of vitamin D has become more important than before (O'Mahony et al. 2011) and in recognition of this, in 2016, the UK Scientific Advisory Committee on Nutrition
(SACN) recommended the national population dietary of 10 µg vitamin D daily for everyone aged 4 years and older (SACN 2016). As there are a limited number of foods naturally enriched with vitamin D (such as egg yolk, oily fish and wild mushroom) (Schmid & Walther 2013), other strategies to improve vitamin D dietary intake are essential.

Vitamin D forms, metabolites and absorption

The two forms of vitamin D, D₂ and D₃, have similar chemical structures apart from vitamin D₂ having an additional methyl group and double bond (Hollis 1984). Humans and animals usually synthesise vitamin D₃ in the skin by converting 7-dehydrocholesterol in the epidermis to pre-vitamin D₃ in response to exposure to ultraviolet B radiation (UVB). Pre-vitamin D₃ then undergoes a temperature-dependent isomerisation to produce vitamin D₃ over approximately 3 days (Holick & Chen 2008). Vitamin D₂ and D₃, obtained from the diet, are absorbed with long-chain triglycerides in the small intestine and then incorporated into chylomicrons and transported via lymph to the circulation (Guo et al. 2018b).

After entering the blood circulation, vitamin D₂ and D₃ follow the same pathways to synthesise the biologically active form of 1, 25(OH)₂ D. There are two hydroxylation reactions: the first reaction occurs in the liver where vitamin D₂ and vitamin D₃ are hydroxylated to 25(OH) D₂ and 25(OH) D₃ by the vitamin D-25-hydroxylase; the second occurs in the kidney where 25(OH) D₂ and 25(OH) D₃ are converted to 1α,25(OH)₂ D₂ and 1α,25(OH)₂ D₃, respectively, by the 25-hydroxyvitamin D-1α-hydroxylase (DeLuca 1974).

Food sources and content of vitamin D

Vitamin D₂ and D₃ can be found in fungi (e.g. mushrooms) and animal-derived foods (e.g. eggs, oily fish), respectively (McCance & Widdowson 2015). In addition, there are significant quantities of the 25(OH) D metabolite in animal-derived foods (Ovesen et al. 2003). Previous
studies (Guo et al. 2017b; Lu et al. 2007; Phillips et al. 2011) have showed that the vitamin D concentrations of these foods can vary significantly between and within species (O’Mahony et al. 2011). For example, Phillips et al. (2011) collected and analysed the vitamin D\textsubscript{3} concentrations in 10 types of mushrooms from retail suppliers in the US, and reported that they were low (0.1-0.3 µg/100 g) in Agaricus bisporus (White Button, Crimini, Portabella) and Enoki, moderate in Shiitake and Oyster (0.4-0.7 µg/100 g), and high in Morel, Chanterelle, Maitake (5.2-28.1 µg/100 g). Furthermore, the vitamin D content of foods may relate to different production systems and the time of the year. For example, our study (Guo et al. 2017b) investigated eggs from three different production systems (organic, free range and indoor) over 5 months and showed a higher vitamin D\textsubscript{3} content in free range eggs (57.2 ± 3.1 µg/kg) and organic eggs (57.2 ± 3.2 µg/kg) compared with indoor eggs (40.2 ± 3.1 µg/kg) ($P <0.001$). A seasonal effect on the vitamin D content of eggs has also been reported by others (Mattila et al. 2011a). The study of Lu et al. (2007) evaluated the vitamin D content of salmon, and found that farmed salmon had only ~25% of the vitamin D content of wild salmon and cooking may also cause detrimental loss of vitamin D. The study of Jakobsen & Knuthsen et al. (2014) investigated the loss/retention of vitamin D during different cooking methods (frying, baking and boiling) in eggs and margarine. The results showed there was 39-45% retention of vitamin D content in eggs and margarine during baking in an oven for 40 minutes, while frying resulted in vitamin D retention of 82-84%. The author concluded that the loss/retention of vitamin D during typical household cooking should be taken into account when calculating the dietary intake of vitamin D.

In general, there are two approaches to fortify foods with vitamin D: 1) ‘direct fortification’ by adding vitamin D into foods and 2) ‘biofortification’ of food by fortifying animal’s diet with vitamin D (Cashman & Kiely 2016). For countries such as the UK where vitamin D fortification of foods is not mandatory (Kiely & Black 2012), populations have to rely on
dietary sources (including supplements) to maintain an adequate vitamin D status when there are limited sunlight. In the UK, the mean daily vitamin D dietary intake (excluding supplements) was 2.9 and 2.5 μg/day for men and women, respectively (NDNS 2008/2009-2011/2012; Bates et al. 2014), which is far less than the current UK dietary reference nutrient intake (RNI) for vitamin D of 10 μg/day (SACN 2016). Therefore, approaches to increase vitamin D dietary intake have become necessary and urgent.

Comparative effectiveness of different forms of vitamin D at raising blood 25(OH) D concentrations

Vitamin D₂ and vitamin D₃

Blood 25(OH) D [the summation of 25(OH) D₂ and 25(OH) D₃] concentration is widely used as a biomarker of vitamin D status (SACN 2016). Early studies reported conflicting results on the relative effectiveness of dietary vitamin D₃ compared with vitamin D₂ for increasing serum/plasma 25(OH) D concentrations (Tripkovic et al. 2017). Tripkovic et al. (2012) conducted a systematic review and meta-analysis comparing the effects of dietary vitamin D₂ and vitamin D₃ on serum 25(OH) D concentrations in humans. Data were included from seven randomised controlled trials (RCTs) and the results showed that vitamin D₃ intake led to a greater absolute change in serum/plasma 25(OH) D levels from baseline than vitamin D₂, with a weighted mean difference of 15.23 (95% CI: 6.12, 24.34; Z=3.28; I²=81%; P=0.001). Recently, a review by Wilson et al. (2017) summarised the evidence to date on the relative effectiveness of vitamin D₃ and vitamin D₂ at raising 25(OH) D concentrations and concluded that most RCTs showed that vitamin D₃ is more effective.

Vitamin D₃ and 25(OH) D₃
Of the few studies performed, most have found that the vitamin D metabolite 25(OH) D\textsubscript{3} given orally increases vitamin D status more efficiently than oral vitamin D\textsubscript{3}, although no consensus has been established for the relative potency of 25(OH) D\textsubscript{3} and vitamin D\textsubscript{3} (Jakobsen 2007). Our recent review (Guo et al. 2018b) summarised the available evidence (Cashman et al. 2012; Catalano et al. 2015; Jetter et al. 2014; Navarro-Valverde et al. 2016) comparing 25(OH) D\textsubscript{3} with vitamin D\textsubscript{3} on serum or plasma 25(OH) D\textsubscript{3} concentrations, and concluded that the relative effectiveness of 25(OH) D\textsubscript{3} to vitamin D\textsubscript{3} ranged from 3.13 to 7.14. These variable results probably reflect differences in study designs and/or characteristics of the investigated subjects. In addition, evidence from available RCTs (Guo et al. 2018b) indicates that 25(OH) D\textsubscript{3} fortified dairy drink resulted in plasma 25(OH) D reach its peak significantly earlier than with vitamin D\textsubscript{3} fortified dairy drink. Thus, supplementation with 25(OH) D\textsubscript{3} might increase vitamin D status more efficiently and effectively than vitamin D\textsubscript{2} and vitamin D\textsubscript{3}. Moreover, since the use of 25(OH) D\textsubscript{3} avoids the need for the liver to convert vitamin D\textsubscript{3} to 25(OH) D\textsubscript{3} it may be of particular value to patients with impaired liver function.

**Food fortification with vitamin D**

*Direct fortification*

In the US and Canada, several common foods, such as milk, orange juices, breakfast cereals, yogurts and cheeses are fortified with vitamin D (Holick et al. 2011). In Europe, vitamin D mandatory and voluntary fortification policies and practice vary from country to country (Spiro & Buttriss 2014). A meta-analysis was performed by Black et al. (2012), which included sixteen RCTs to evaluate the efficacy of vitamin D food fortification for improving vitamin D status. The results showed a mean intake of vitamin D of 11 µg/day from fortified foods (range 3-25 µg/day) increased serum/plasma 25(OH) D by 19.4 nmol/L (95% CI: 13.9-24.9), which corresponded to a 1.2 nmol/L (95% CI: 0.72, 1.68) increase in serum/plasma 25(OH) D for
each 1 µg ingested. Thus, vitamin D direct fortification could be an effective strategy to increase vitamin D status in the general UK population.

In the US and Canada, much of the vitamin D intake is from fortified foods (Fulgoni et al. 2011; Langlois et al. 2010). The major fortified foods contributing to vitamin D intake in these countries are fluid milk, ready-to-eat cereals and margarine (Calvo et al. 2004; Feldman et al. 2011). The study by Langlois et al. (2010) estimated vitamin D status among 5306 individuals aged 6-79 years in the 2007-2009 Canadian Health Measures Survey and showed that the mean 25(OH) D concentration was 67.7 nmol/L, and that 4% and 10% of the population had vitamin D deficiency (<27.5 nmol/L) and inadequacy (<37.5 nmol/L), respectively. In addition, subjects who consumed vitamin D fortified milk had higher 25(OH) D concentrations than non-consumers. In addition, voluntary fortification of foods with vitamin D has occurred in Finland since 2003 (Pilz et al. 2018), and the data from the Finnish Health 2011 Survey showed that mean serum 25(OH) D increased from 47.6 nmol/L in year 2000 to 65.4 nmol/L in 2011 (Jaaskelainen et al. 2017). However, a recent review (Calvo & Whiting 2013) questioned the adequacy of vitamin D fortified foods in the US and Canada to meet the needs of all race, gender and age groups. Furthermore, a review by Kiely et al. (2012) pointed out well-designed sustainable fortification strategies are needed to take account for diversity in food consumption patterns. In the UK, the food fortification policy was effective in preventing rickets in the 1950s; however, the mandatory vitamin D fortification policy was banned when over-fortification of some milk products led to cases of hypercalcaemia in young children (British Pediatric Association 1956). More research is needed to explore the safety of vitamin D fortification, including the range of products and doses of vitamin D added in each.

Biofortification
Biofortification of vitamin D is an alternative strategy to increase vitamin D intakes in countries and regions where policies and practices limit use of ‘direct fortification’.

Our previous review provides an overview of recent vitamin D biofortification studies (Guo et al. 2018b), and found that the amount of vitamin D₃ and 25(OH) D₃ in eggs, fish and milk increases in response to vitamin D₃ supplementation of the diets of hens, fish and cows. However, evidence relating to 25(OH) D₃ supplementation of animals’ diets is very limited, with the only available data for hens (Guo et al. 2018b). Interestingly, egg enrichment studies (Duffy et al. 2017; Mattila et al. 2011b) showed that supplementing hens’ diets with 25(OH) D₃ results in an increase in the 25(OH) D₃ concentration, but not vitamin D₃, of the egg yolk. Thus, foods biofortified or fortified with either vitamin D₃ or 25(OH) D₃ are likely to have a variable effect on human vitamin D status (Mattila et al. 2011b).

Our recent milk biofortification study (Guo et al. 2018a) used a total of 60 dairy cows randomised to vitamin D₃ or 25(OH) D₃ dietary supplementing treatments, within the maximum permitted European Union (EU) vitamin D₃ concentration (2 mg/day vitamin D₃) for feed. The results showed that supplementing dairy cows’ feed with 25(OH) D₃ significantly increased circulating plasma concentrations of 25(OH) D₃ in the cows. However, there was also no significant effect of the treatment on milk 25(OH) D₃ concentrations (P=0.193), the mean 25(OH) D₃ concentrations for non-fortified and 25(OH) D₃ dietary treatments were 869 and 1001 ng/kg, respectively. In addition, the vitamin D concentration (100-3,300 ng/kg) of the biofortified milk was negligible and far less than the current UK vitamin D recommended intake of 10 µg/day (SACN 2016). In the future, more studies are needed to explore which forms and doses of vitamin D added to animal diets, within the bounds of EU regulation (EC 2017; EFSA 2012), including those of fish, may have the greatest impact on human dietary quality.
Evidence from human intervention studies with 25(OH) D₃ fortified foods

Evidence of the effect of 25(OH) D₃ fortified food on increasing vitamin D status is limited. We were the first to compare the effects of dairy drinks fortified with either 20 µg 25(OH) D₃ or 20 µg vitamin D₃ on changes in human 24-hour vitamin D status (Guo et al. 2017a). The results showed plasma 25(OH) D₃ was significantly higher after the 25(OH) D₃ fortified dairy drink compared with the vitamin D₃ fortified dairy drink and control (non-fortified dairy drink), which was reflected in the 1.5-fold and 1.8-fold greater incremental area under the curve of plasma 25(OH) D₃ for the 0-8 hour response, respectively. However, we did not investigate the long-term effects of consuming the 25(OH) D₃ and vitamin D₃ fortified dairy drinks.

Hayes et al. (2016) conducted an 8-week RCT to compare the effects of consuming vitamin D₃ or 25(OH) D₃ biofortified eggs (7 per week for 8 weeks), obtained from feeding hens with the maximum concentration of vitamin D₃ or 25(OH) D₃ lawfully allowed in their diets, with a control treatment (≤ 2 commercial eggs/week), on winter serum 25(OH) D concentrations in healthy adults. At the 8 week follow-up in winter the vitamin D status of the subjects who consumed the vitamin D₃ or 25(OH) D₃ biofortified eggs was maintained [50.4 nmol/L (SD=21.4) and 49.2 nmol/L (SD=16.5) for vitamin D₃ and 25(OH) D₃ group, respectively], while the control group’s vitamin D status significantly decreased over winter (-6.4 ± 6.7 nmol/L). In contrast with our study (Guo et al. 2017a), there was no significant difference between vitamin D₃ and 25(OH) D₃ biofortified egg consumption on the participants’ serum 25(OH) D concentrations. The reason is unknown, but maybe because baseline vitamin D status (mean 46.2 nmol/L) was much higher than our study (mean 31.7 nmol/L), and vitamin D dose (3.5-4.5 µg/egg) for fortified eggs (Hayes et al. 2016) was only 20% of ours (20 µg/day) (Guo et al. 2017a).

25(OH) D₃ supplementation and human health
As an alternative strategy to increase vitamin D status, it is possible that supplementation with 25(OH) D₃ may benefit human health more than with vitamin D₃, although the evidence is limited. A study of Bischoff-Ferrari et al. (2012) provided 20 µg/day of 25(OH) D₃ or vitamin D₃ to 20 healthy postmenopausal women over 4 months [mean baseline serum 25(OH) D concentration was 42 nmol/L]. The results showed 25(OH) D₃ supplementation resulted in a more immediate and sustained increase of serum 25(OH) D concentrations than vitamin D₃ supplementation. The mean 25(OH) D concentration increased to 221 nmol/L and 99 nmol/L for 25(OH) D₃ and vitamin D₃ supplementation, respectively. In addition, 25(OH) D₃ supplementation was found, on average, to result in a 2.8-fold increased odds of maintained or improved lower extremity function (OR=2.79, 95% CI: 1.18-6.58), and a 5.7 mmHg decrease in systolic blood pressure compared with vitamin D₃ (P=0.0002). In another study, Jean et al. (2008) provided 10-30 µg/day 25(OH) D₃ to haemodialysis patients for 6 months, and the results showed vitamin D status increased from 30 nmol/L to 126 nmol/L, and 25(OH) D₃ supplementation corrected their excess bone turnover.

A review by Brandi & Minisola (2013) summarised the available evidence in this area and concluded that for populations that have specific conditions (such as long-lasting vitamin D osteomalacia, liver failure, iatrogenic inhibition of liver 25-hydroxylases, inactivating mutations of genes encoding liver 25-hydroxylasese, kidney failure with elevated PTH, nephrosis, transplanted patients, male hypogonadism), supplementation with 25(OH) D₃ may prove to be preferable to vitamin D₃. The reasons might be because 25(OH) D₃ avoids the need for hepatic metabolism of vitamin D₃ to 25(OH) D₃, which results in 25(OH) D₃ more quickly entering the blood circulation (Holick 1995; Ross et al. 2011).

Currently, vitamin D₂ and vitamin D₃ are legally permitted to be added to foods, but addition of 25(OH) D₃ is not (EC No 1925/2006). Future studies should focus on better defining the
Conclusions and future directions

Vitamin D deficiency and insufficiency have become global problems, especially where sunlight is limited by latitude, cultural reasons or lifestyle (Hilger et al. 2014). The UK government advisory committee, SACN, recommends an intake of 10 µg/day of vitamin D for the UK general population (SACN 2016). However, it is a great challenge to meet this recommendation from solely natural dietary sources and uptake of supplements tends to be low. Two potential strategies to increase vitamin D content of food are direct fortification and biofortification via animal diet supplementation. However, evidence from RCTs is limited on the effect of vitamin D fortified foods on human vitamin D status and human health. The available evidence suggests that the vitamin D metabolite, 25(OH) D₃, might be more efficient than vitamin D₂ and D₃ at raising serum or plasma 25(OH) D₃ concentrations in both general healthy subjects and clinical patients. In addition, 25(OH) D₃ may have an advantage of improving the health of certain clinical patients, although the evidence for this is limited. Therefore, 25(OH) D₃ fortified foods (including direct fortification and biofortification) should be further explored in the future, and additional RCTs should be conducted to investigate the effect of 25(OH) D₃ fortified foods on vitamin D status and human health in both healthy subjects and clinical patients.

Conflict of interest

The authors have no conflict of interest to disclose.
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