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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 D₃ fortified foods

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1 **Abstract**

2 Hypovitaminosis D is widespread throughout the world. The cutaneous production of vitamin
3 D through sunlight can be limited by several factors (*e.g.* skin pigmentation, sunscreen usage
4 and, increasingly, indoor lifestyle). Thus, diet has become an important strategy to increase
5 vitamin D intake and status. However, there are a limited number of foods (*e.g.* eggs, oily fish
6 and wild mushroom) naturally enriched with vitamin D, and concentrations can vary
7 significantly between and within species. Therefore, the need for vitamin D fortified foods
8 (including via direct fortification and biofortification) to support adequacy of vitamin D status
9 [blood 25-hydroxivitamin D (25(OH) D)] is a corollary of several limitations to synthesise
10 vitamin D from sunlight. Ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) can be
11 found in some mushrooms and animal-derived foods, respectively. Evidence has shown
12 vitamin D₃ is more effective than vitamin D₂ at raising 25(OH) D blood concentrations. The
13 vitamin D metabolite, 25(OH) D₃, is present in animal-derived foods (*e.g.* meat, eggs and fish),
14 and several intervention trials have shown 25(OH) D₃ to be more effective at raising blood
15 25(OH) D concentrations than vitamin D₃. In addition, 25(OH) D₃ supplements may prove to
16 be preferable to vitamin D₃ for patients with certain clinical conditions. However, there is
17 limited evidence on the effect of 25(OH) D₃ fortified foods on human vitamin D status and
18 health. Therefore, long-term randomised controlled trials to evaluate the effect of 25(OH) D₃
19 fortified foods on vitamin D status are needed for both the general population and patients with
20 certain conditions.

21

22 **Key words**

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

24 **Introduction**

25 Vitamin D is a lipid soluble vitamin that acts as a hormone (Nair & Maseeh 2012), which
26 generally refers to ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) (Tripkovic *et*
27 *al.* 2012). Vitamin D₂ and vitamin D₃ are produced by fungi and the skin of vertebrates,
28 respectively (Wacker & Holick 2013). The role of vitamin D in musculoskeletal health is well
29 established (Wolff *et al.* 2008). Recently, vitamin D deficiency has been suggested to be
30 associated with several non-musculoskeletal health outcomes, such as cardiovascular disease,
31 certain cancers and type 2 diabetes, although mechanisms are not clear (Wang *et al.* 2017).
32 Vitamin D status is assessed by measuring the blood concentration of circulating 25-
33 hydroxyvitamin D (25(OH) D) (Holick 2009). Widespread hypovitaminosis D is now
34 acknowledged (Hilger *et al.* 2014), although there is some dispute about the thresholds for
35 vitamin D deficiency and insufficiency (Spiro & Buttriss 2014). In the UK, vitamin D
36 deficiency is defined as 25(OH) D <25 nmol/L (SACN 2016). The UK *National Diet and*
37 *Nutritional Survey (NDNS)* reported that in 2008-2012 24% men and 21.7% of women (aged
38 19-64 years) had vitamin D deficiency (Bates *et al.* 2014). With seasonal variation, the
39 prevalence of hypovitaminosis D in the UK was alarmingly high during winter and spring. A
40 cross-sectional study conducted in the UK by Hypponen and Power (2007) reported that during
41 the winter and spring months 25(OH) D concentrations were <25 nmol/L, <40 nmol/L and <75
42 nmol/L in 15.5%, 46.6% and 87.1% of participants, respectively. There are several additional
43 contributors to hypovitaminosis D, such as skin pigmentation, sunscreen usage, and an
44 increasingly indoor lifestyle, all of which reduce the cutaneous production of vitamin D (Holick
45 2004). Furthermore, vitamin D supplement can also contribute to vitamin D intake, however,
46 uptake of supplements tends to be low (Hennessy *et al.* 2017; Datta *et al.* 2016). As a result,
47 dietary intake of vitamin D has become more important than before (O'Mahony *et al.* 2011)
48 and in recognition of this, in 2016, the UK Scientific Advisory Committee on Nutrition

49 (SACN) recommended the national population dietary of 10 µg vitamin D daily for everyone
50 aged 4 years and older (SACN 2016). As there are a limited number of foods naturally enriched
51 with vitamin D (such as egg yolk, oily fish and wild mushroom) (Schmid & Walther 2013),
52 other strategies to improve vitamin D dietary intake are essential.

53

54 **Vitamin D forms, metabolites and absorption**

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

63 After entering the blood circulation, vitamin D₂ and D₃ follow the same pathways to
64 synthesise the biologically active form of 1, 25(OH)₂D. There are two hydroxylation reactions:
65 the first reaction occurs in the liver where vitamin D₂ and vitamin D₃ are hydroxylated to
66 25(OH) D₂ and 25(OH) D₃ by the vitamin D-25-hydroxylase; the second occurs in the kidney
67 where 25(OH) D₂ and 25(OH) D₃ are converted to 1α,25(OH)₂ D₂ and 1α,25(OH)₂ D₃,
68 respectively, by the 25-hydroxyvitamin D-1α-hydroxylase (DeLuca 1974).

69

70 **Food sources and content of vitamin D**

71 Vitamin D₂ and D₃ can be found in fungi (*e.g.* mushrooms) and animal-derived foods (*e.g.* eggs,
72 oily fish), respectively (McCance & Widdowson 2015). In addition, there are significant
73 quantities of the 25(OH) D metabolite in animal-derived foods (Ovesen *et al.* 2003). Previous

74 studies (Guo *et al.* 2017b; Lu *et al.* 2007; Phillips *et al.* 2011) have showed that the vitamin D
75 concentrations of these foods can vary significantly between and within species (O'Mahony *et*
76 *al.* 2011). For example, Phillips *et al.* (2011) collected and analysed the vitamin D₂
77 concentrations in 10 types of mushrooms from retail suppliers in the US, and reported that they
78 were low (0.1-0.3 µg/100 g) in *Agaricus bisporus* (White Button, Crimini, Portabella) and
79 Enoki, moderate in Shiitake and Oyster (0.4-0.7 µg/100 g), and high in Morel, Chanterelle,
80 Maitake (5.2-28.1 µg/100 g). Furthermore, the vitamin D content of foods may relate to
81 different production systems and the time of the year. For example, our study (Guo *et al.* 2017b)
82 investigated eggs from three different production systems (organic, free range and indoor) over
83 5 months and showed a higher vitamin D₃ content in free range eggs (57.2 ± 3.1 µg/ kg) and
84 organic eggs (57.2 ± 3.2 µg/ kg) compared with indoor eggs (40.2 ± 3.1 µg/ kg) (*P* <0.001). A
85 seasonal effect on the vitamin D content of eggs has also been reported by others (Mattila *et*
86 *al.* 2011a). The study of Lu *et al.* (2007) evaluated the vitamin D content of salmon, and found
87 that farmed salmon had only ~ 25% of the vitamin D content of wild salmon and cooking may
88 also cause detrimental loss of vitamin D. The study of Jakobsen & Knuthsen *et al.* (2014)
89 investigated the loss/ retention of vitamin D during different cooking methods (frying, baking
90 and boiling) in eggs and margarine. The results showed there was 39-45% retention of vitamin
91 D content in eggs and margarine during baking in an oven for 40 minutes, while frying resulted
92 in vitamin D retention of 82-84%. The author concluded that the loss/ retention of vitamin D
93 during typical household cooking should be taken into account when calculating the dietary
94 intake of vitamin D.

95 In general, there are two approaches to fortify foods with vitamin D: 1) 'direct fortification'
96 by adding vitamin D into foods and 2) 'biofortification' of food by fortifying animal's diet with
97 vitamin D (Cashman & Kiely 2016). For countries such as the UK where vitamin D
98 fortification of foods is not mandatory (Kiely & Black 2012), populations have to rely on

99 dietary sources (including supplements) to maintain an adequate vitamin D status when there
100 are limited sunlight. In the UK, the mean daily vitamin D dietary intake (excluding
101 supplements) was 2.9 and 2.5 µg/day for men and women, respectively (NDNS 2008/2009-
102 2011/2012; Bates *et al.* 2014)), which is far less than the current UK dietary reference nutrient
103 intake (RNI) for vitamin D of 10 µg/day (SACN 2016). Therefore, approaches to increase
104 vitamin D dietary intake have become necessary and urgent.

105

106 **Comparative effectiveness of different forms of vitamin D at raising blood 25(OH) D** 107 **concentrations**

108 *Vitamin D₂ and vitamin D₃*

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

121

122 *Vitamin D₃ and 25(OH) D₃*

123 Of the few studies performed, most have found that the vitamin D metabolite 25(OH) D₃ given
124 orally increases vitamin D status more efficiently than oral vitamin D₃, although no consensus
125 has been established for the relative potency of 25(OH) D₃ and vitamin D₃ (Jakobsen 2007).
126 Our recent review (Guo *et al.* 2018b) summarised the available evidence (Cashman *et al.* 2012;
127 Catalano *et al.* 2015; Jetter *et al.* 2014; Navarro-Valverde *et al.* 2016) comparing 25(OH) D₃
128 with vitamin D₃ on serum or plasma 25(OH) D₃ concentrations, and concluded that the relative
129 effectiveness of 25(OH) D₃ to vitamin D₃ ranged from 3.13 to 7.14. These variable results
130 probably reflect differences in study designs and/or characteristics of the investigated subjects.
131 In addition, evidence from available RCTs (Guo *et al.* 2018b) indicates that 25(OH) D₃ fortified
132 dairy drink resulted in plasma 25(OH) D reach its peak significantly earlier than with vitamin
133 D₃ fortified dairy drink. Thus, supplementation with 25(OH) D₃ might increase vitamin D status
134 more efficiently and effectively than vitamin D₂ and vitamin D₃. Moreover, since the use of
135 25(OH) D₃ avoids the need for the liver to convert vitamin D₃ to 25(OH) D₃ it may be of
136 particular value to patients with impaired liver function.

137

138 **Food fortification with vitamin D**

139 *Direct fortification*

140 In the US and Canada, several common foods, such as milk, orange juices, breakfast cereals,
141 yogurts and cheeses are fortified with vitamin D (Holick *et al.* 2011). In Europe, vitamin D
142 mandatory and voluntary fortification policies and practice vary from country to country (Spiro
143 & Buttriss 2014). A meta-analysis was performed by Black *et al.* (2012), which included
144 sixteen RCTs to evaluate the efficacy of vitamin D food fortification for improving vitamin D
145 status. The results showed a mean intake of vitamin D of 11 µg/day from fortified foods (range
146 3-25 µg/day) increased serum/plasma 25(OH) D by 19.4 nmol/L (95% CI: 13.9-24.9), which
147 corresponded to a 1.2 nmol/L (95% CI: 0.72, 1.68) increase in serum/plasma 25(OH) D for

148 each 1 µg ingested. Thus, vitamin D direct fortification could be an effective strategy to
149 increase vitamin D status in the general UK population.

150 In the US and Canada, much of the vitamin D intake is from fortified foods (Fulgoni *et al.*
151 2011; Langlois *et al.* 2010). The major fortified foods contributing to vitamin D intake in these
152 countries are fluid milk, ready-to-eat cereals and margarine (Calvo *et al.* 2004; Feldman *et al.*
153 2011). The study by Langlois *et al.* (2010) estimated vitamin D status among 5306 individuals
154 aged 6- 79 years in the 2007- 2009 *Canadian Health Measures Survey* and showed that the
155 mean 25(OH) D concentration was 67.7 nmol/L, and that 4% and 10% of the population had
156 vitamin D deficiency (<27.5 nmol/L) and inadequacy (<37.5 nmol/L), respectively. In addition,
157 subjects who consumed vitamin D fortified milk had higher 25(OH) D concentrations than
158 non-consumers. In addition, voluntary fortification of foods with vitamin D has occurred in
159 Finland since 2003 (Pilz *et al.* 2018), and the data from the *Finnish Health 2011 Survey* showed
160 that mean serum 25(OH) D increased from 47.6 nmol/L in year 2000 to 65.4 nmol/L in 2011
161 (Jaaskelainen *et al.* 2017). However, a recent review (Calvo & Whiting 2013) questioned the
162 adequacy of vitamin D fortified foods in the US and Canada to meet the needs of all race,
163 gender and age groups. Furthermore, a review by Kiely *et al.* (2012) pointed out well-designed
164 sustainable fortification strategies are needed to take account for diversity in food consumption
165 patterns. In the UK, the food fortification policy was effective in preventing rickets in the
166 1950s; however, the mandatory vitamin D fortification policy was banned when over-
167 fortification of some milk products led to cases of hypercalcaemia in young children (British
168 Pediatric Association 1956). More research is needed to explore the safety of vitamin D
169 fortification, including the range of products and doses of vitamin D added in each.

170

171 *Biofortification*

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

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

183 Our recent milk biofortification study (Guo *et al.* 2018a) used a total of 60 dairy cows
184 randomised to vitamin D₃ or 25(OH) D₃ dietary supplementing treatments, within the maximum
185 permitted European Union (EU) vitamin D₃ concentration (2 mg/day vitamin D₃) for feed. The
186 results showed that supplementing dairy cows’ feed with 25(OH) D₃ significantly increased
187 circulating plasma concentrations of 25(OH) D₃ in the cows. However, there was also no
188 significant effect of the treatment on milk 25(OH) D₃ concentrations ($P=0.193$), the mean
189 25(OH) D₃ concentrations for non-fortified and 25(OH) D₃ dietary treatments were 869 and
190 1001 ng/kg, respectively. In addition, the vitamin D concentration (100-3,300 ng/kg) of the
191 biofortified milk was negligible and far less than the current UK vitamin D recommended
192 intake of 10 µg/day (SACN 2016). In the future, more studies are needed to explore which
193 forms and doses of vitamin D added to animal diets, within the bounds of EU regulation (EC
194 2017; EFSA 2012), including those of fish, may have the greatest impact on human dietary
195 quality.

196

197 *Evidence from human intervention studies with 25(OH) D₃ fortified foods*

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

206 Hayes *et al.* (2016) conducted an 8-week RCT to compare the effects of consuming vitamin
207 D₃ or 25(OH) D₃ biofortified eggs (7 per week for 8 weeks), obtained from feeding hens with
208 the maximum concentration of vitamin D₃ or 25(OH) D₃ lawfully allowed in their diets, with a
209 control treatment (≤ 2 commercial eggs/week), on winter serum 25(OH) D concentrations in
210 healthy adults. At the 8 week follow-up in winter the vitamin D status of the subjects who
211 consumed the vitamin D₃ or 25(OH) D₃ biofortified eggs was maintained [50.4 nmol/L
212 (SD=21.4) and 49.2 nmol/L (SD=16.5) for vitamin D₃ and 25(OH) D₃ group, respectively],
213 while the control group's vitamin D status significantly decreased over winter (-6.4 ± 6.7
214 nmol/L). In contrast with our study (Guo *et al.* 2017a), there was no significant difference
215 between vitamin D₃ and 25(OH) D₃ biofortified egg consumption on the participants' serum
216 25(OH) D concentrations. The reason is unknown, but maybe because baseline vitamin D status
217 (mean 46.2 nmol/L) was much higher than our study (mean 31.7 nmol/L), and vitamin D dose
218 (3.5-4.5 µg/egg) for fortified eggs (Hayes *et al.* 2016) was only 20% of ours (20 µg/day) (Guo
219 *et al.* 2017a)..

220

221 **25(OH) D₃ supplementation and human health**

222 As an alternative strategy to increase vitamin D status, it is possible that supplementation with
223 25(OH) D₃ may benefit human health more than with vitamin D₃, although the evidence is
224 limited. A study of Bischoff-Ferrari *et al.* (2012) provided 20 µg/day of 25(OH) D₃ or vitamin
225 D₃ to 20 healthy postmenopausal women over 4 months [mean baseline serum 25(OH) D
226 concentration was 42 nmol/L]. The results showed 25(OH) D₃ supplementation resulted in a
227 more immediate and sustained increase of serum 25(OH) D concentrations than vitamin D₃
228 supplementation. The mean 25(OH) D concentration increased to 221 nmol/L and 99 nmol/L
229 for 25(OH) D₃ and vitamin D₃ supplementation, respectively. In addition, 25(OH) D₃
230 supplementation was found, on average, to result in a 2.8-fold increased odds of maintained or
231 improved lower extremity function (OR=2.79, 95% CI: 1.18-6.58), and a 5.7 mmHg decrease
232 in systolic blood pressure compared with vitamin D₃ ($P=0.0002$). In another study, Jean *et al.*
233 (2008) provided 10-30 µg/day 25(OH) D₃ to haemodialysis patients for 6 months, and the
234 results showed vitamin D status increased from 30 nmol/L to 126 nmol/L, and 25(OH) D₃
235 supplementation corrected their excess bone turnover.

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

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

246 long-term effects of 25(OH) D₃ fortified foods on vitamin D status and human health, compared
247 to vitamin D₃ and vitamin D₂.

248

249 **Conclusions and future directions**

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

266

267 **Conflict of interest**

268 The authors have no conflict of interest to disclose.

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