

Effects of vitamin D₂-fortified bread v. supplementation with vitamin D₂ or D₃ on serum 25-hydroxyvitamin D metabolites: an 8-week randomised-controlled trial in young adult Finnish women

Suvi T. Itkonen^{1*}, Essi Skaffari¹, Pilvi Saaristo¹, Elisa M. Saarnio¹, Maijaliisa Erkkola¹, Jette Jakobsen², Kevin D. Cashman³ and Christel Lamberg-Allardt¹

¹Calcium Research Unit, Department of Food and Environmental Sciences, Division of Nutrition, PO Box 66, University of Helsinki, FI-00014 Helsinki, Finland

²Division of Food Technology, National Food Institute, Technical University of Denmark, 2860 Soborg, Denmark

³School of Food and Nutritional Sciences, Cork Centre for Vitamin D and Nutrition Research, University College Cork, T12 E31 Cork, Republic of Ireland

(Submitted 23 September 2015 – Final revision received 29 December 2015 – Accepted 5 January 2016 – First published online 11 February 2016)

Abstract

There is a need for food-based solutions for preventing vitamin D deficiency. Vitamin D₃ (D₃) is mainly used in fortified food products, although the production of vitamin D₂ (D₂) is more cost-effective, and thus may hold opportunities. We investigated the bioavailability of D₂ from UV-irradiated yeast present in bread in an 8-week randomised-controlled trial in healthy 20–37-year-old women (*n* 33) in Helsinki (60°N) during winter (February–April) 2014. Four study groups were given different study products (placebo pill and regular bread = 0 µg D₂ or D₃/d; D₂ supplement and regular bread = 25 µg D₂/d; D₃ supplement and regular bread = 25 µg D₃/d; and placebo pill and D₂-biofortified bread = 25 µg D₂/d). Serum 25-hydroxyvitamin D₂ (S-25(OH)D₂) and serum 25-hydroxyvitamin D₃ (S-25(OH)D₃) concentrations were measured at baseline, midpoint and end point. The mean baseline total serum 25-hydroxyvitamin D (S-25(OH)D = S-25(OH)D₂ + S-25(OH)D₃) concentration was 65.1 nmol/l. In repeated-measures ANCOVA (adjusted for baseline S-25(OH)D as total/D₂/D₃), D₂-bread did not affect total S-25(OH)D (*P* = 0.707) or S-25(OH)D₃ (*P* = 0.490), but increased S-25(OH)D₂ compared with placebo (*P* < 0.001). However, the D₂ supplement was more effective than bread in increasing S-25(OH)D₂ (*P* < 0.001). Both D₂ and D₃ supplementation increased total S-25(OH)D compared with placebo (*P* = 0.030 and *P* = 0.001, respectively), but D₂ supplementation resulted in lower S-25(OH)D₃ (*P* < 0.001). Thus, D₂ from UV-irradiated yeast in bread was not bioavailable in humans. Our results support the evidence that D₂ is less potent in increasing total S-25(OH)D concentrations than D₃, also indicating a decrease in the percentage contribution of S-25(OH)D₃ to the total vitamin D pool.

Key words: Vitamin D₂; Vitamin D₃; Bioavailability; 25-Hydroxyvitamin D; 25-Hydroxyvitamin D₂; 25-Hydroxyvitamin D₃; UV-irradiated yeast

Vitamin D₃ (D₃) is synthesised in skin exposed to UVB radiation in sunlight. However, in Northern Europe, including Finland, exposure to sunlight is limited or non-existent in winter⁽¹⁾. Between November and March, dietary or supplementary vitamin D intake is essential to maintain adequate serum 25-hydroxyvitamin D (S-25(OH)D) concentrations. There are only a few natural dietary sources of vitamin D. Fish and egg yolks contain significant amounts of D₃ (cholecalciferol), and some wild mushrooms are a source of vitamin D₂ (D₂, ergocalciferol). Thus, vitamin D fortification of certain foods has been suggested as one strategy to improve vitamin D status of Northern European populations⁽²⁾, but also that such strategies use a range of foods to accommodate dietary diversity⁽³⁾.

Although D₃ is the most commonly used vitamin in fortified products in Europe currently⁽²⁾, use of D₂ as a fortificant may also have some advantages. For example, it may be important for vegetarians, vegans and those with cultural considerations who would not benefit from animal-derived sources of vitamin D, whether as foods or supplements. The production of plant-derived D₂ could be more cost-effective than production of D₃. Finally, although the potential for biofortification of animal-derived foods has been proposed as food-based solutions for vitamin D deficiency^(3,4), there is also potential for D₂-biofortified foods such as those arising from UVB irradiation of mushrooms and baker's yeast, which have been shown to stimulate their endogenous D₂ content. As yet, these are not very common in

Abbreviations: D₂, vitamin D₂; D₃, vitamin D₃; S-25(OH)D, serum 25-hydroxyvitamin D; S-25(OH)D₂, serum 25-hydroxyvitamin D₂; S-25(OH)D₃, serum 25-hydroxyvitamin D₃; S-PTH, serum parathyroid hormone.

* **Corresponding author:** S. T. Itkonen, email suvi.itkonen@helsinki.fi

the European Union. While potentially of major importance in addressing vitamin D deficiency, a key consideration is whether these food-based sources of D₂ are effective and whether they might have a negative impact on serum 25-hydroxyvitamin D₃ (S-25(OH)D₃) concentrations. Recently, Logan *et al.*⁽⁵⁾ showed that supplementation of New Zealand adults with D₂ (25 µg/d) for 25 weeks in winter increased serum 25-hydroxyvitamin D₂ (S-25(OH)D₂) but produced a 9 nmol/l greater decline in S-25(OH)D₃ compared with placebo. This contributed to the finding that end point total S-25(OH)D concentrations were 21 nmol/l lower in participants receiving D₂ compared with those receiving D₃ (25 µg/d) among whom total S-25(OH)D concentrations remained unchanged during winter. Data on the impact of food-derived D₂ on S-25(OH)D₃ are limited⁽²⁾. A systematic review reported that supplemental D₃ is more effective in raising total S-25(OH)D concentrations than supplemental D₂⁽⁶⁾. The half-life of D₂ has been found to be shorter after a bolus dose in vitamin D-deficient subjects⁽⁷⁾. A reduction in S-25(OH)D₃ despite significant increases in S-25(OH)D₂ may have physiological consequences. For instance, D₃ seems to be more efficient than D₂ in decreasing serum parathyroid hormone (S-PTH) levels in vitamin D-deficient subjects⁽⁷⁾.

Thus, we aimed to investigate the bioavailability of D₂ from UV-irradiated baking yeast present in bread in terms of efficacy of raising S-25(OH)D₂ and total S-25(OH)D (i.e. S-25(OH)D₂ + S-25(OH)D₃) as well as its possible effects on S-25(OH)D₃ and S-PTH concentrations compared with D₂ or D₃ supplementation in a randomised placebo-controlled trial in young, healthy females during winter in Finland. The UV-irradiated baking yeast is known to be safe and suitable for baking and has been approved by the Food and Drug Administration (FDA) in the USA and by the European Food Safety Authority (EFSA) in Europe^(8,9).

Methods

Subjects

The study was conducted as part of the European Union-funded research project ODIN (Food-based solutions for optimal vitamin D nutrition and health through the lifecycle, FP7-613977-ODIN; www.odin-vitd.eu). Specifically, it was a part of Work Package 5 within the ODIN project where the overall aim was to investigate the potential advantages and limitations of use of novel food-based solutions for the prevention of vitamin D deficiency. D₂-biofortified yeast was one of these novel food-based solutions. The intervention study 'BreaD' was carried out at the Viikki Campus of the University of Helsinki (60°N) in Finland over 8 weeks from February to April 2014, when the availability of UVB radiation of sunlight is insufficient for endogenous vitamin D production, and S-25(OH)D concentrations are expected to be the lowest⁽¹⁾. Study volunteers were mainly recruited from the University Campus areas via advertisements for personnel, student mailing lists and the Internet. This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Helsinki Uusimaa Hospital District Ethics Committees.

Written informed consent was obtained from all subjects. The study was registered on ClinicalTrials.gov (www.ClinicalTrials.gov; NCT025003).

Inclusion criteria were good general health (no continuous medication), Caucasian origin, BMI <30 kg/m² and aged between 20 and 40 years. Exclusion criteria were S-25(OH)D >100 nmol/l, diagnosed coeliac disease or cereal allergy, a vacation in a sunny place during the study period or within the preceding 2 months, regular use of vitamin D supplements >15 µg/d and pregnancy or breast-feeding during the study.

Study protocol

The subjects were blindly randomised into four study groups (Fig. 1), with ten to eleven subjects in each group and similar mean total S-25(OH)D concentrations between the groups. During the 8-week trial, three groups were given regular wheat bread and either +a daily placebo pill (group 1), +a daily D₂ supplement (group 2) or +a daily D₃ supplement (group 3), and a further group was given D₂-containing wheat bread +a daily placebo pill (group 4). The average daily portion of bread was 87 g (three slices of bread/d), similar to the daily bread consumption of Finnish females in the nationally representative FINDIET 2012 Study⁽¹⁰⁾. The daily dose of vitamin D, 25 µg, corresponds to 2.5 times the national Finnish recommendation for vitamin D intake (10 µg) for that age group⁽¹¹⁾. Overnight fasting serum samples were collected between 07.30 and 10.00 hours at baseline (week 0), midpoint (week 4) and end point (week 8) of the study.

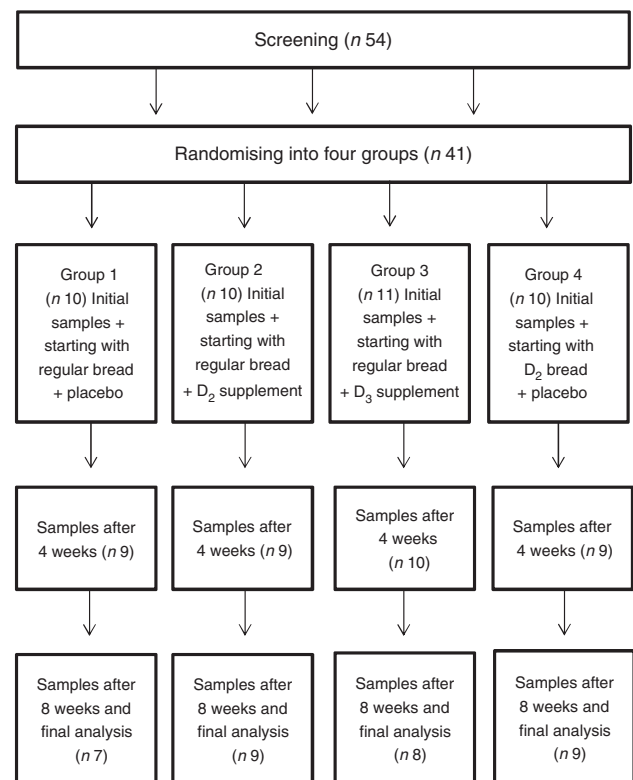


Fig. 1. Flow chart of the study. D₂, vitamin D₂; D₃, vitamin D₃.

The supplements/placebos (see below) were delivered to the subjects at the beginning of the study. The frozen breads (see below) were transported weekly from the bakery to the research unit and stored at -20°C . The breads were delivered to the subjects once or twice a week. If the subjects took the weekly allotment in a single delivery, they were advised to store the breads in the freezer at home. The subjects were asked to maintain their habitual diets and exercise habits during the study period. In addition to study breads, the subjects were allowed to eat other breads if they wished to consume more than the requested three slices. The subjects kept a daily record for 8 weeks on all study breads and supplements consumed, and compliance was calculated based on these records and cumulative pill counts at the end of the study.

Habitual vitamin D and Ca intakes of subjects were assessed by a validated semi-quantitative FFQ⁽¹²⁾. The FFQ was administered by trained interviewers at the baseline and the end point of the study. The use of vitamin and mineral supplements (other than the study supplements) was ascertained by a separate questionnaire at the beginning of the study. The vitamin D content of the foodstuffs was obtained from the Finnish food composition database Fineli[®], which is developed and continuously updated by the Finnish National Institution of Health and Welfare (www.fineli.fi).

Breads and supplements

The study breads were provided by a local bakery 'Marian Konditoria' (Vantaa, Finland). The breads were baked in one batch and stored at -20°C until delivery to the participants. The D₂-biofortified bread (which will be referred to as D₂-bread hereafter) was baked with 'UV-treated baker's yeast' (Lallemand SAS), a regular baker's yeast (*Saccharomyces cerevisiae*), in which D₂ had been produced from ergosterol by UVB light exposure. The product is approved by the FDA in the USA and by the EFSA in Europe^(8,9). The D₂ content of the D₂-bread (n 4) was $30.2\ \mu\text{g}/100\ \text{g}$, thereby providing the participants $26.3\ \mu\text{g}$ D₂ in their daily portion of 87 g bread (analysed by HPLC). The regular bread was baked with regular baker's yeast, and showed no content of vitamin D ($<0.3\ \mu\text{g}$ D₂/100 g, n 4). The supplements contained $24.4\ \mu\text{g}$ D₂ per each D₂ capsule (n 3) (Helhetshälsa AB) and $25.0\ \mu\text{g}$ D₃ per each D₃ pill (Oy Verman Ab). The HPLC method used for the analyses of D₂ was run accredited according to ISO17025 at Technical University of Denmark⁽¹³⁾. The standard deviation for the method was 6.5%, and the detection limit was $0.3\ \mu\text{g}/100\ \text{g}$.

Biochemical analyses

The total S-25(OH)D concentrations of the screening samples were assessed 1–2 weeks before the trial by OCEIA immunoenzymometric assay (IDS) at the Department of Food and Environmental Sciences, University of Helsinki. Reproducibility of the analysis was ensured by adhering to the Vitamin D External Quality Assessment Scheme (DEQAS; Charing Cross Hospital, London, UK; <http://deqas.kpmd.co.uk/>). The laboratory received the DEQAS proficiency certificate in 2012. Inter-assay and intra-assay CV were 2.7 and 3.2%, respectively. The S-25(OH)D

concentrations in the trial samples (i.e. baseline, midpoint and end point) were analysed by the Vitamin D Research Group at University College Cork in Ireland using a liquid chromatography-tandem MS (LC-MS/MS) core analytical platform for the ODIN project. This LC-MS/MS method measures S-25(OH)D₂ and S-25(OH)D₃ separately, and total S-25(OH)D concentrations were calculated based on the summation of these values. The inter-assay and intra-assay CV for the analyses were <5 and <6 %, respectively, for both metabolites⁽¹⁴⁾. The quality and accuracy of S-25(OH)D analysis by the LC-MS/MS in our laboratory is monitored on an ongoing basis by participation in the DEQAS. In addition, the Vitamin D Research Group method is certified under the Centers for Disease Control and Prevention Vitamin D Standardization Certification Program (http://www.cdc.gov/labstandards/pdf/hs/CDC_Certified_Vitamin_D_Procedures.pdf). Serum intact parathyroid hormone (S-iPTH) concentrations were analysed by the immunoluminescence-based method using Immulite1000 (Siemens Healthcare Diagnostics), and serum Ca (S-Ca) concentrations were analysed by a photometric method using Konelab20 automatic analyser (Thermo Clinical Labsystems Oy) at the Department of Food and Environmental Sciences, University of Helsinki. For S-iPTH analyses, the inter-assay and intra-assay CV were <8.0 and <5.5 %, respectively, and for S-Ca <4.6 and <4.6 %, respectively.

Sample size estimation and statistical analyses

A power calculation based on total S-25(OH)D concentrations was performed to estimate the number of subjects needed to detect a 25 (SD 15) nmol/l change between the groups after intervention, assuming a power of 90% ($\alpha=0.05$). This calculation estimated that a sample size of eight per intervention group would be adequate (thirty-two in total); however, this number was increased by 25% to account for possible dropouts. Anticipating that some potential volunteers would have relatively high S-25(OH)D concentrations, we decided to include a total of fifty-four subjects who qualified for participation and screen them for total S-25(OH)D concentration. The forty-one subjects with the lowest S-25(OH)D concentrations were accepted as participants.

Statistical analysis was performed using SPSS Statistics version 22 (IBM). Data are presented as mean values and standard deviations, and in figures as means with their standard errors. The normality and homogeneity of the data were verified and log-transformed to improve normality, if needed. All tests were considered significant at $P<0.05$. Correlations between S-25(OH)D and vitamin D intake variables and potential confounding factors were assessed by Spearman's correlation coefficients. One-way ANOVA was used to assess differences in background variables between intervention groups; non-parametric tests were used for non-normally distributed variables (i.e. vitamin D intake variables). *Post hoc* tests were carried out with least significant difference for the normally distributed variables. If the variables were not normally distributed, the Kruskal–Wallis test was used. Repeated-measures ANOVA and repeated-measures ANCOVA were used to assess the impact of the intervention on total S-25(OH)D, S-25(OH)D₂ (non-normally

distributed; log-transformed), S-25(OH)D₃, S-PTH and S-Ca as well as changes in S-25(OH)D, S-25(OH)D₂, S-25(OH)D₃, S-PTH and S-Ca concentrations over time. In ANCOVA, the baseline total S-25(OH)D/S-25(OH)D₂/S-25(OH)D₃/S-PTH/S-Ca concentration was used as a covariate. Comparisons between groups were carried out with contrasts.

Results

Subject characteristics

Altogether, thirty-seven of forty-one recruited subjects completed the study. For the final analysis, we excluded two subjects with baseline total S-25(OH)D concentrations >100 nmol/l (as per exclusion criteria, even though their screening samples had total S-25(OH)D concentrations <100 nmol/l) (all the analyses were also carried out including these two persons, and no changes in the results were found) – one subject who used an own vitamin D supplement (in addition to the trial supplement) containing 20 µg/d and one subject who discontinued the use of own vitamin D supplement, which resulted in a significant decrease in total S-25(OH)D concentration at the end point for that subject. Thus, the number of subjects included in the final analyses was thirty-three. Baseline characteristics of these thirty-three subjects are presented in Table 1. The intervention groups did not differ from each other significantly in relation to background characteristics, except for S-PTH, where the D₂-bread intervention group had a significantly higher S-PTH concentration than the placebo ($P=0.022$) or the D₃ supplement group ($P=0.008$) (Table 1). The subjects were healthy, aged between 20 and 37 years and did not have continuous medication, except for hormonal contraception (six subjects). None of the subjects reported smoking regularly. In all, twelve of the subjects were allowed to continue taking their own vitamin D supplements ($\leq 15 \mu\text{g/d}$) during the study period, but the mean vitamin D intake from own supplements was only 2.7 µg/d and this small

supplemental intake should not have greatly affected the results. The mean compliance of the study products among the subjects was 96.7 %, and no differences were present between intervention groups ($P>0.05$) (data not shown).

Mean baseline total S-25(OH)D concentration among the study subjects was 65.1 nmol/l, and it did not differ significantly among groups. The average total vitamin D intake was sufficient (12.2 µg/d) based on the national Finnish recommendation of vitamin D intake of 10 µg/d⁽¹¹⁾. The average Ca intake was also adequate at 1217 mg/d (recommended intake 800 mg/d)⁽¹¹⁾ and was similar to that of the general population in Finland⁽¹⁰⁾.

Effects on total serum 25-hydroxyvitamin D (Fig. 2(a))

The responses to treatment differed among the groups over time ($P=0.003$): D₃ was more effective in increasing total S-25(OH)D than placebo ($P=0.001$) or D₂-bread ($P=0.002$). The D₂ supplement increased total S-25(OH)D concentrations relative to placebo ($P=0.030$), but only borderline relative to D₂-bread ($P=0.050$). The mean changes in the D₂ supplement group and the D₃ supplement group were +9.6 nmol/l (+14.7%) and +17.0 nmol/l (+26.2%), respectively. In placebo and D₂-bread groups, total S-25(OH)D remained at baseline levels. No other differences between groups emerged ($P>0.05$) (repeated-measures ANCOVA, adjusted for baseline total S-25(OH)D concentration).

Effects on serum 25-hydroxyvitamin D₃ (Fig. 2(b))

A difference among the groups in response to supplementation was present over time ($P<0.001$): D₃ supplementation increased S-25(OH)D₃ the most (compared with all the other groups, $P<0.001$). The S-25(OH)D₃ concentration decreased in the D₂ supplement group relative to the D₂-bread ($P=0.001$) and placebo groups ($P<0.001$). The mean changes in the D₂ supplement group and D₃ supplement group were -21.7 nmol/l

Table 1. Background characteristics of the subjects in the four intervention groups and differences between the groups (Mean values and standard deviations; numbers and percentages)

	Placebo (n 7)		D ₂ supplement (n 9)		D ₃ supplement (n 8)		D ₂ -bread (n 9)		All participants (n 33)		P (ANOVA)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Age (years)	24.9	4.8	25.6	4.2	30.8	3.7	27.6	6.0	27.2	5.1	0.089
BMI (kg/m ²)	21.7	1.5	22.4	1.5	23.0	3.1	21.8	2.8	22.2	2.3	0.661
Baseline serum 25-hydroxyvitamin D (nmol/l)	66.2	18.6	63.5	11.3	66.6	14.8	64.6	15.1	65.1	14.2	0.973
Baseline serum 25-hydroxyvitamin D ₃ (nmol/l)	63.1	18.8	59.8	10.9	61.6	15.2	61.1	14.6	61.3	14.2	0.977
Baseline serum 25-hydroxyvitamin D ₂ (nmol/l)	3.1	1.4	3.8	2.0	5.1	6.0	3.5	2.0	3.9	3.3	0.947*
Baseline serum parathyroid hormone (ng/l)	32.8	12.8	40.5	16.7	30.6	7.6	49.7	15.7	39.0	15.3	0.035†
Baseline serum Ca (mmol/l)	2.34	0.08	2.28	0.13	2.36	0.07	2.32	0.07	2.32	0.09	0.375
Frequency of supplement users											
n		2		3		4		3		12	
%		29		33		50		33		36	
Vitamin D intake from own supplement (µg/d)	2.1	3.6	2.7	4.2	4.2	5.7	1.8	3.4	2.7	4.2	0.771*
Dietary vitamin D intake (food) (µg/d) during the study	7.2	2.5	10.2	6.6	10.4	4.0	10.1	3.5	9.6	4.5	0.374*
Total vitamin D intake (food + supplement) (µg/d) during the study	9.3	3.8	12.8	9.9	14.5	5.4	11.9	5.3	12.2	6.6	0.313*
Ca intake (mg/d) during the study	1040	528	1272	602	1403	661	1135	349	1217	536	0.585

D₂, vitamin D₂; D₃, vitamin D₃.

* P values from non-parametric tests.

† In between-group comparison in the D₂-bread group concentration is higher compared with placebo ($P=0.022$) and D₃ supplement group ($P=0.008$).

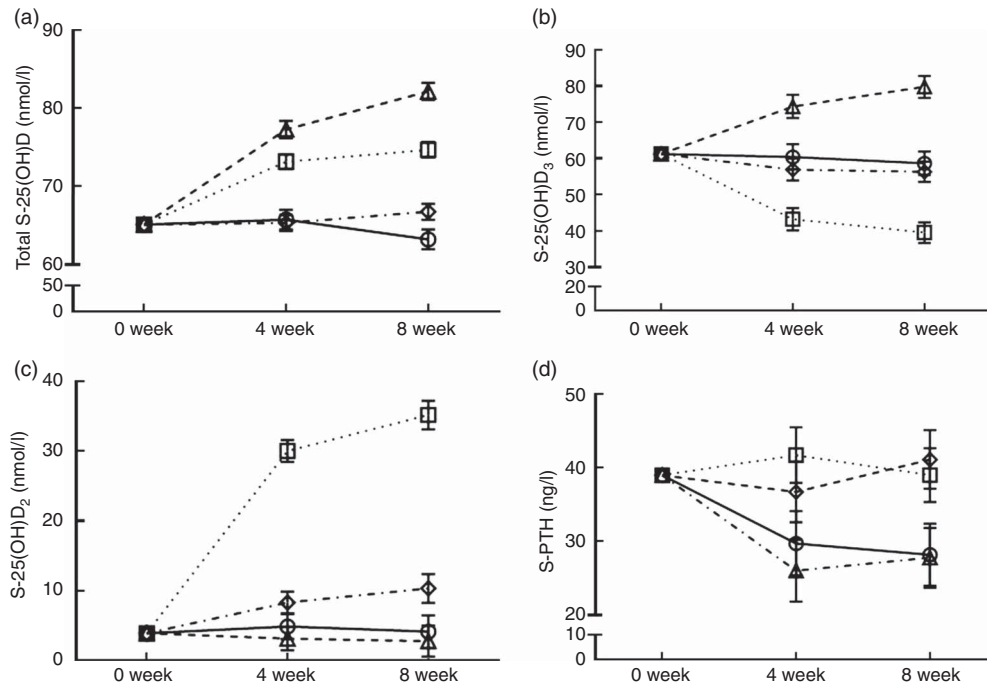


Fig. 2. Response of total serum 25-hydroxyvitamin D (S-25(OH)D) (a), serum 25-hydroxyvitamin D₃ (S-25(OH)D₃) (b), serum 25-hydroxyvitamin D₂ (S-25(OH)D₂) (c) and serum parathyroid hormone (S-PTH) (d) concentration to vitamin D supplementation over the 8-week study period. Time points are at 4-week intervals, representing mean values at each time point, error bars represent standard errors. In repeated-measures ANCOVA, the differences were significant: total S-25(OH)D ($P=0.003$), S-25(OH)D₃ ($P<0.001$), S-25(OH)D₂ ($P<0.001$) and S-PTH ($P=0.018$). The significances of between-group comparisons (contrasts) are reported in the text. The values are adjusted for baseline total S-25(OH)D/S-25(OH)D₃/S-25(OH)D₂/S-PTH concentrations. D₂, vitamin D₂; D₃, vitamin D₃; —◇—, D₂-bread; —▲—, D₃ supplement; —□—, D₂ supplement; —○—, placebo.

(−35.4%) and +18.5 nmol/l (+30.3%), respectively. S-25(OH)D₃ concentration remained at similar levels throughout the study in the D₂-bread and placebo groups. No other changes between the groups were observed ($P<0.05$) (repeated-measures ANCOVA, adjusted for baseline S-25(OH)D₃ concentration).

Effects on serum 25-hydroxyvitamin D₂ (Fig. 2(c))

The effect of supplementation on S-25(OH)D₂ concentrations differed among the groups over time ($P<0.001$). The D₂ supplement was the most effective in increasing S-25(OH)D₂ concentration (compared with all the other groups, $P<0.001$), and D₂-bread resulted in a higher S-25(OH)D₂ concentration than placebo or D₃ supplement (P for both <0.001). The mean changes in the D₂ supplement group and D₂-bread group were +31.3 and +6.4 nmol/l, respectively. No changes relative to baseline concentrations in the placebo or D₃ supplement group emerged. No other significant differences in between-group comparisons were evident ($P>0.05$) (repeated-measures ANCOVA, adjusted for baseline S-25(OH)D₂ concentration).

Effects on serum parathyroid hormone (Fig. 2(d)) and serum calcium

The responses of S-PTH concentrations to supplementation differed among the groups over time ($P=0.018$). The D₂ supplement and D₂-bread groups remained at baseline levels throughout the study. S-PTH concentrations decreased in the

D₃ supplement group compared with the D₂-bread ($P=0.021$) and D₂ supplement groups ($P=0.006$). Relative to the D₂ group, S-PTH decreased in the placebo group ($P=0.019$). The mean changes in placebo and D₃ supplement groups were −10.8 nmol/l (−27.0%) and −11.2 nmol/l (−28.7%), respectively. Between other groups, no differences were observed ($P>0.05$). No impact of intervention on S-Ca concentrations during the trial was present ($P>0.05$, results not shown) (repeated-measures ANCOVA, adjusted for baseline S-PTH/S-Ca concentration).

Discussion

Some studies have shown that D₃-fortified breads improve S-25(OH)D status in different population groups^(15,16), and some D₃-fortified breads are currently on the market in Finland as well. The aim of our study was to determine whether UVB-activated D₂ of yeast added to bread, as a new source of vitamin D, could improve S-25(OH)D status in an attempt to find a cheaper and more ecological alternative for improving vitamin D intake and status in the general population. In our 8-week randomised-controlled trial (RCT) in young adult females during winter in Finland, the daily ingestion of bread containing 25 µg of D₂ derived from UVB-irradiated baker's yeast only very modestly increased mean S-25(OH)D₂ concentration (by 6.4 nmol/l), whereas mean total S-25(OH)D concentration did not increase nor were there any changes in mean S-25(OH)D₃ or S-PTH concentrations. In contrast, daily

supplementation of young adult females with 25 µg of D₂ as a capsule increased mean S-25(OH)D₂ and total S-25(OH)D concentrations (by 31.3 and 9.6 nmol/l, respectively) over the same time frame. These findings indicate a very low bioavailability of D₂ from the UVB-irradiated yeast in bread. Interestingly, the daily supplementation with 25 µg of D₂ decreased S-25(OH)D₃ concentrations (by 21.7 nmol/l, on average), which led to the mean increase in total S-25(OH)D concentration being less than that achieved by supplementation with an equivalent amount of D₃ (by 17.0 nmol/l less) over the same time frame. A decreasing effect of D₂ supplementation on S-25(OH)D₃ concentrations has also been noted in earlier studies^(5,6). The Tripkovic *et al.*⁽⁶⁾ meta-analysis of vitamin D supplemental studies suggested that no proper data existed with regard to food-derived D₂.

The present study, to our knowledge, was the first to test D₂-enriched bread in humans and showed a poor bioavailability of D₂ from UVB-irradiated yeast. Hohman *et al.*⁽¹⁷⁾, in their rat experiment, showed a significant increase in total S-25(OH)D in young growing rats fed UVB-irradiated yeast incorporated into bread. Beyond the species and life-stage differences between that study and our present study, the rats in their study⁽¹⁷⁾ that were highly vitamin D deficient were in the bread intervention phase (7 weeks of a lead-in diet containing 2.5% of American Institute of Nutrition-recommended vitamin D intake for growing rodents, leading to a mean plasma 25(OH)D of approximately 2 nmol/l entering the study), whereas the healthy young adults in our RCT were of relatively good vitamin D status (mean S-25(OH)D, 65.1 nmol/l at baseline), which is very much in line with the current situation at a national level in Finland⁽¹⁸⁾. Thus, the baseline vitamin D status may impact on the S-25(OH)D response of subjects to increased D₂ intake in the form of bread made from UV-irradiated yeast. Also of interest, the plasma 25(OH)D response of the rats in the Hohman *et al.*⁽¹⁷⁾ study to D₂ (from UVB-irradiated yeast) was only half of that achieved by rats given an equivalent amount of D₃ (provided in crystalline form in the diet, not included in the bread). Unfortunately, the study lacked a group provided with D₂ as a supplement, which could have shown a difference in the bioavailability between sources of D₂. The reason for the lack of efficacy of the UVB-activated D₂ of yeast in raising S-25(OH)D₂ and total S-25(OH)D in the healthy young adult females in the current study is not clear. It is interesting to note that, although UV-irradiated mushrooms have been effective in increasing S-25(OH)D concentrations and bone growth in rats⁽¹⁹⁾, the results in human studies are contradictory^(20–23). A recent meta-analysis of data from randomised-controlled studies on the effectiveness of UV-exposed edible mushrooms on raising serum total S-25(OH)D showed that the effectiveness was only evident when the baseline vitamin D status of participants was inadequate (mean S-25(OH)D < approximately 50 nmol/l) and not when status was high⁽²⁴⁾. The increased D₂ from consumption of UV mushrooms led to a significant increase in S-25(OH)D₂ but a partial reduction in S-25(OH)D₃, which reduced the overall response of total S-25(OH)D. Of note, in the two studies with high status (mean S-25(OH)D >70 nmol/l), the increase and decrease of S-25(OH)D₂ and S-25(OH)D₃, respectively, were of an equal magnitude such that

there was no net change in total S-25(OH)D. The meta-analysis also pointed to the potential for the pre-treatment of the mushrooms explaining the differences among studies⁽²⁴⁾. In the present study, the D₂ group, which had a much greater increase in S-25(OH)D₂, compared with the D₂-bread group (31.3 *v.* 6.4 nmol/l, respectively), had a significant reduction in S-25(OH)D₃ (–21.7 nmol/l), whereas the D₂-bread group had no significant change. These findings may point to the possibility that the baking process of the bread, which, although not affecting the D₂ content of the bread, may have influenced the bioavailability of D₂ in the bread. Thus, it is possible that the digestibility of the yeast preparation was low and did not allow liberation of the D₂, something that will require further investigation.

In the present study, D₃ supplementation (25 µg/d) increased S-25(OH)D₃ and total S-25(OH)D concentrations, as expected, and had no effect on S-25(OH)D₂. As seen in previous studies, an inverse relationship between S-PTH and total S-25(OH)D concentrations was present⁽¹⁾. In our study, supplementation with D₃ resulted in decreases in S-PTH concentrations. Nevertheless, this effect was not observed in the D₂ supplement group, even though the total S-25(OH)D concentration increased. This observation is in line with that of Romagnoli *et al.*⁽⁷⁾, who found that D₃ decreased S-PTH, whereas D₂ had no effect on S-PTH. Further, no effects of supplementation on S-Ca concentrations were perceived; however, measurement of serum ionised Ca, the active form of Ca, would have provided more accurate data on possible changes in Ca homeostasis.

Overall, our results support the contention that D₂ appears to be a less effective alternative than D₃ in improving vitamin D status^(7,25,26). Treatment with D₂ has been suggested to cause a decrease in the percentage contribution of D₃ to the total vitamin D pool that is available for hydroxylation to the active metabolite – 1,25-dihydroxyvitamin D – resulting in a decrease in S-25(OH)D₃⁽²⁷⁾. However, it remains uncertain whether D₂ and D₃ and their metabolites are biologically equivalent and whether a competitive situation exists⁽²⁷⁾. Studies have shown that supplementation with D₂ contributed to lower S-25(OH)D₃ concentrations than D₃ supplementation, even though total S-25(OH)D concentrations increased with doses of 25–50 µg/d for 8–25 weeks^(5,28,29). Moreover, in a cross-sectional study, higher S-25(OH)D₂ has been associated with lower S-25(OH)D₃ and further lower concentrations of the active metabolite of D₃, S-1,25(OH)D₃⁽²⁷⁾. It is important to note that different analytical methods for 25(OH)D may affect the results⁽³⁰⁾, as all of the methods are not able to separate S-25(OH)D₂ and S-25(OH)D₃. However, in our study and in the above-mentioned studies, LC-MS/MS methods, which detect both forms distinctly, were used^(27–29).

The mean baseline total S-25(OH)D concentration among the study participants was above the 50 nmol/l recommended by the Institute of Medicine⁽³¹⁾, being similar to the concentration in the general Finnish population⁽¹⁸⁾. On the basis of power calculations, we aimed to find an increase of 25 nmol/l in total S-25(OH)D in the treatment groups. In our study, the largest difference in S-25(OH)D was present between the D₃ supplementation group and the placebo group (18.9 nmol/l), and did not reach the aimed level. Thus, the fairly high baseline S-25(OH)D concentrations and adequate vitamin D intake may

have hidden a stronger effect of the intervention on total S-25(OH)D concentrations, which could have been found with lower baseline concentrations. At present, due to the improved vitamin D consciousness in the Finnish population, it is difficult to recruit subjects with low S-25(OH)D for intervention studies. In the cross-sectional population study, the FINDIET 2012 Study, 56% of the female participants used vitamin D supplements⁽¹⁰⁾. The relatively good S-25(OH)D status is probably due to the fortification policy in Finland and the increased use of supplementary vitamin D.

Conclusions

The D₂ from the biofortified bread did not appear to possess good bioavailability in humans. The reason for this is obscure, possibly caused by the baking process of the bread or a potentially undigestible form of D₂ in the yeast preparation. However, our results strengthen the data that D₂ supplementation decreases S-25(OH)D₃ concentrations, indicating possible replacement of S-25(OH)D₃ by S-25(OH)D₂ in biological actions of vitamin D. Possibly affecting the results is that the baseline total S-25(OH)D concentrations of the subjects were fairly satisfactory.

Acknowledgements

The authors thank Dr Heli T Viljakainen for statistical advice and technician Anu Heiman-Lindh for laboratory analyses in Helsinki.

This work was supported by funding received by the authors from the European Commission under its Seventh Framework Programme (ODIN; grant agreement no. 613977). Lallemand SAS provided the UVB yeast and study breads. Study supporters had no role in the design, analysis or writing of this article.

The author contributions are as follows: C. L.-A., J. J. and K. D. C. are grant holders. S. T. I., E. S., P. S., M. E. and C. L.-A. designed the study. E. S., P. S. and E. M. S. collected the data. J. J. was responsible for the bread and supplement vitamin D analyses. K. D. C. was responsible for the S-25(OH)D analyses in Cork. S. T. I. performed the statistical analyses and drafted the manuscript. All authors reviewed and approved the final draft of the manuscript.

The authors have no conflicts of interest to declare.

References

- Lamberg-Allardt C, Brustad M, Meyer HE, *et al.* (2013) Vitamin D – a systematic review for the 5th edition of the Nordic Nutrition Recommendations. *Food Nutr Res* **57**, 10.3402/fnr.v57i0.22671.
- Black IJ, Seamans KM, Cashman KD, *et al.* (2012) An updated systematic review and meta-analysis of the efficacy of vitamin D food fortification. *J Nutr* **142**, 1102–1108.
- Cashman KD & Kiely M (2015) Tackling inadequate vitamin D intakes – is fortification of dairy products with vitamin D enough? *Endocrine* **51**, 38–46.
- Cashman KD (2015) Vitamin D: dietary requirements and food fortification as a means of helping achieve adequate vitamin D status. *J Steroid Biochem Mol Biol* **148**, 19–26.
- Logan VF, Gray AR, Peddie MC, *et al.* (2013) Long-term vitamin D₃ supplementation is more effective than vitamin D₂ in maintaining serum 25-hydroxyvitamin D status over the winter months. *Br J Nutr* **109**, 1082–1088.
- Tripkovic L, Lambert H, Hart K, *et al.* (2012) Comparison of vitamin D₂ and vitamin D₃ supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am J Clin Nutr* **95**, 1357–1364.
- Romagnoli E, Mascia ML, Cipriani C, *et al.* (2008) Short and long-term variations in serum calcitropic hormones after a single very large dose of ergocalciferol (vitamin D₂) or cholecalciferol (vitamin D₃) in the elderly. *J Clin Endocrinol Metab* **93**, 3015–3022.
- Food and Drug Administration (2012) *Food additives permitted for direct addition to food for human consumption; vitamin D₂ bakers yeast*. *Federal Register* **77**, 168. <http://www.gpo.gov/fdsys/pkg/FR-2012-08-29/html/2012-21353.htm> (accessed June 2015).
- European Food Safety Agency (2014) Scientific opinion on the safety of vitamin D-enriched UV-treated baker's yeast. *EFSA J* **12**, 3520.
- Helldán A, Raulio S, Kosola M, *et al.* (2013) *The National FINDIET 2012 Survey. Reports of Institution of Health and Welfare, 16/2013*. Tampere, Finland: Juvenes Print.
- National Nutrition Council (2014) *Suomalaiset ravitsemussuosituksset – Terveystä ruoasta (Finnish Nutrition Recommendations – Health from Food)*. Helsinki: Juvenes Print (in Finnish).
- Itkonen ST, Erkkola M, Skaffari E, *et al.* (2015) Development and validation of an interview-administered food frequency questionnaire for assessment of vitamin D and calcium intakes in Finnish women. *Br J Nutr*, (Epublication ahead of print version 21 December 2015).
- Kristensen HL, Rosenqvist E & Jakobsen J (2012) Increase of vitamin D₂ by UV-B exposure during the growth phase of white button mushroom (*Agaricus bisporus*). *Food Nutr Res* **56**, 7114–7121.
- Cashman KD, Kiely M, Kinsella M, *et al.* (2013) Evaluation of Vitamin D Standardization Program protocols for standardizing serum 25-hydroxyvitamin D data: a case study of the program's potential for national nutrition and health surveys. *Am J Clin Nutr* **97**, 1235–1242.
- Natri A-M, Salo P, Vikstedt T, *et al.* (2006) Bread fortified with cholecalciferol increases the serum 25-hydroxyvitamin D concentration in women as effectively as a cholecalciferol supplement. *J Nutr* **136**, 123–127.
- Mocanu V & Vieth R (2013) Three-year follow-up of serum 25-hydroxyvitamin D, parathyroid hormone, and bone mineral density in nursing home residents who had received 12 months of daily bread fortification with 125 mug of vitamin D₃. *Nutr J* **12**, 137.
- Hohman EE, Martin BR, Lachlik PJ, *et al.* (2011) Bioavailability and efficacy of vitamin D₂ from UV-irradiated yeast in growing, vitamin D-deficient rats. *J Agric Food Chem* **59**, 2341–2346.
- Cashman KD, Dowling K, Skrabakova Z, *et al.* (2015) Standardizing serum 25-hydroxyvitamin D data from four Nordic population samples using the Vitamin D Standardization Program protocols: shedding new light on vitamin D status in Nordic individuals. *Scand J Clin Lab Invest* **75**, 549–561.
- Calvo MS, Babu US, Garthoff LGH, *et al.* (2013) Vitamin D₂ from light-exposed edible mushrooms is safe, bioavailable and effectively supports bone growth in rats. *Osteoporos Int* **24**, 197–207.
- Urbain P, Singler F, Ihorst G, *et al.* (2011) Bioavailability of vitamin D₂ from UV-B-irradiated button mushrooms in healthy adults deficient in serum 25-hydroxyvitamin D: a randomized controlled trial. *Eur J Clin Nutr* **65**, 965–971.

21. Stephensen CB, Zerofsky M, Burnett DJ, *et al.* (2012) Ergocalciferol from mushrooms or supplements consumed with a standard meal increases 25-hydroxyergocalciferol but decreases 25-hydroxycholecalciferol in the serum of healthy adults. *Nutr J* **142**, 1246–1252.
22. Stepien M, O'Mahony L, O'Sullivan A, *et al.* (2013) Effect of supplementation with vitamin D₂-enhanced mushrooms on vitamin D status in healthy adults. *J Nutr Sci* **2**, e29.
23. Mehrotra A, Calvo MS, Beelman RB, *et al.* (2014) Bioavailability of vitamin D₂ from enriched mushrooms in prediabetic adults: a randomized controlled trial. *Eur J Clin Nutr* **68**, 1154–1160.
24. Cashman KD, Kiely M, Seamans KM, *et al.* (2016) Effect of ultraviolet light-exposed mushrooms on vitamin D status: liquid chromatography-tandem mass spectrometry re-analysis of biobanked sera from a randomized controlled trial and a systematic review plus meta-analysis. *J Nutr* (In the Press).
25. Jones G (2013) Extrarenal vitamin D activation and interactions between vitamin D₂, vitamin D₃, and vitamin D analogs. *Annu Rev Nutr* **33**, 23–44.
26. Houghton LA & Vieth R (2006) The case against ergocalciferol (vitamin D₂) as a vitamin supplement. *Am J Clin Nutr* **84**, 694–697.
27. Swanson CM, Nielson CM, Shrestha S, *et al.* (2014) Higher 25(OH)D₂ is associated with lower 25(OH)D₃ and 1,25(OH)₂D₃. *J Clin Endocrinol Metab* **99**, 2736–2744.
28. Biancuzzo RM, Clarke N, Reitz RE, *et al.* (2013) Serum concentrations of 1,25-dihydroxyvitamin D₂ and 1,25-dihydroxyvitamin D₃ in response to vitamin D₂ and vitamin D₃ supplementation. *J Clin Endocrinol Metab* **98**, 973–979.
29. Lehmann U, Hirche F, Stangl GI, *et al.* (2013) Bioavailability of vitamin D₂ and D₃ in healthy volunteers, a randomized placebo-controlled trial. *J Clin Endocrinol Metab* **98**, 4339–4345.
30. El-Hajj Fuleihan G, Bouillon R, Clarke B, *et al.* (2015) Serum 25-hydroxyvitamin D levels: variability, knowledge gaps, and the concept of desirable range. *J Bone Miner Res* **30**, 1119–1133.
31. Ross AC, Manson JE, Abrams SA, *et al.* (2011) The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* **96**, 53–58.