

Bioavailability and Efficacy of Vitamin D₂ from UV-Irradiated Yeast in Growing, Vitamin D-Deficient Rats

Emily E. Hohman,[†] Berdine R. Martin,[†] Pamela J. Lachcik,[†] Dennis T. Gordon,[‡] James C. Fleet,[†] and Connie M. Weaver^{*,†}

[†]Department of Foods & Nutrition, 700 W. State Street, Purdue University, West Lafayette, Indiana 47907, United States

[‡]North Dakota State University, Fargo, North Dakota 58102, United States

ABSTRACT: New food sources are needed to bridge the gap between vitamin D intake and recommended intake. We assessed the bioavailability and efficacy of vitamin D in an 8 week dose–response study of bread made with vitamin D₂-rich yeast compared to vitamin D₃ in growing, vitamin D-deficient rats. Plasma 25-hydroxyvitamin D (25OHD) levels increased in a curvilinear, dose-dependent manner with both forms of vitamin D, but rats fed vitamin D₂-rich yeast achieved lower levels than rats fed vitamin D₃. Rats fed the highest doses of vitamin D had significantly greater ($p < 0.05$) trabecular BMC, BMD, bone volume, and connectivity density, and greater midshaft total cross-sectional area, compared to rats on the vitamin D-deficient diets, with no significant difference due to vitamin D source. Vitamin D₂-rich yeast baked into bread is bioavailable and improves bone quality in vitamin D-deficient animals.

KEYWORDS: Vitamin D, fortification, bioavailability, bone, rats

INTRODUCTION

Vitamin D is a major regulator of calcium absorption¹ and therefore a determinant of bone mineral acquisition. Emerging research also suggests that vitamin D may also play a role in risk for a number of chronic diseases.² Optimal vitamin D status, as measured by serum or plasma 25-hydroxyvitamin D (25OHD) levels, has not been clearly defined, but some have proposed that as many as 70% of the U.S. population may have insufficient vitamin D status.³

Vitamin D₃ can be produced endogenously in the skin via UVB irradiation-induced conversion of 7-dehydrocholesterol. However, cutaneous synthesis of vitamin D may not be sufficient to meet vitamin D needs in populations with limited sun exposure, resulting in a dependency on dietary vitamin D. Unfortunately, few foods are naturally rich in vitamin D, and fortified foods are relatively limited⁴ and often inconsistent in their vitamin D content.⁵ A recent analysis of vitamin D intake from NHANES 2005–2006 data showed that most Americans did not reach the adequate intake (AI) of vitamin D from diet alone.⁶ Among adolescents aged 9–19 y, only 24–53% achieved the AI, 200 IU/d, from vitamin D in foods, and among adults over age 50 y, fewer than 10% achieved the AI, 400–600 IU/d, from food alone. These percentages increased somewhat when vitamin D supplements were considered, but $\geq 20\%$ of the U.S. population still failed to meet their vitamin D requirements. With the recent increase in vitamin D requirements to 600 IU/d and 800 IU/d for those over age 70,⁷ the gap widens. New food sources of vitamin D could address this gap.

Milk is the staple food fortified with vitamin D in the United States. In the past, vitamin D content of fortified milks has often been inconsistent and below the level specified on the label,⁵ with the variation partly attributed to the addition of the fortificant in local dairies without adequate quality control. Recently published data showed that consistency of fortification has improved but

that 16% of sampled milks still had vitamin D levels lower than the amount on the label.⁸ Furthermore, at levels of 100 international units (IU)/cup, even if Americans consumed the three cups of milk per day recommended by the dietary guidelines,⁹ this source of vitamin D alone is insufficient to bring serum 25OHD values to optimal levels for most Americans.¹⁰ Other fortified foods have even lower levels of vitamin D per serving.⁴

Novel sources of vitamin D are being evaluated. Fungi, such as yeast and mushrooms, produce vitamin D₂ via UVB irradiation of ergosterol, making them naturally rich sources of vitamin D. Vitamin D₂ from UV-treated mushrooms have been found to increase serum 25OHD and bone mineral density (BMD) in rats.¹¹ Vitamin D₂-rich UV-treated yeast can be incorporated into bread, a widely consumed food item. On average, Americans consume 79 g or approximately 3 slices of yeast bread per day,¹² making it an ideal new source of vitamin D. We investigated the bioavailability of vitamin D₂ from bread made with vitamin D₂-rich yeast by determining its dose–response relationship on plasma 25OHD compared to crystalline vitamin D₃ in growing, vitamin D-deficient rats. Additionally, we assessed the ability of crystalline vitamin D₃ and bread made with vitamin D₂-rich yeast to improve bone properties and microarchitecture as a functional outcome measurement of vitamin D status.

MATERIALS AND METHODS

Animals. Four week old male Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN). Rats were housed in individual cages in a temperature- and humidity-controlled room with a 12 h on/off

Received: December 6, 2010

Accepted: January 19, 2011

Revised: January 6, 2011

Published: February 18, 2011

light cycle. Clear UV tube guards (Pegasus Associates, Beaver Falls, PA) were placed on the lights to prevent exposure to UVB light. Food and water were provided ad libitum. All procedures were approved by Purdue University's Animal Care and Use Committee.

Diets. Upon arrival, rats were fed a vitamin D-deficient diet based on the AIN93G formulation,¹³ modified to contain 25 IU vitamin D₃/kg feed (Research Diets, New Brunswick, NJ). The experimental diets were also based on the AIN93G formulation but were designed to contain 25, 100, 200, or 1000 IU crystalline vitamin D₃ or vitamin D₂ from D₂-rich yeast baked into bread/kg diet, for a total of 8 experimental diets. For the bread-based diets, five loaves of bread were prepared with vitamin D₂-rich yeast (Lallemand Yeast). The vitamin D contents of the breads ranged from 0 to 4.28 IU vitamin D₂/g. Approximately 350 g of bread/kg diet was used in place of corn starch as a source of carbohydrate. Different ratios of the five loaves were used to achieve the desired vitamin D level for each diet. All of the diets were isocaloric and contained identical percentages of protein, fat, and carbohydrate. The vitamin D content of the diets and the bread used to make the diets was analyzed by HPLC in the laboratory of Dr. Michael Holick.¹⁴

Study Design. Rats were maintained on the vitamin D-deficient diet for 7 weeks to establish vitamin D deficiency. At the end of 7 weeks, five rats were sacrificed, and blood was collected to confirm vitamin D deficiency. The remaining rats were randomized to the eight dietary treatments ($n = 10/\text{group}$), which were fed for 8 additional weeks. At the end of 8 weeks on the experimental diets, all rats were euthanized by an overdose of CO₂. Blood was collected from the dorsal aorta into heparinized vacutainers, centrifuged to separate plasma, and stored at -80°C prior to analysis. The right femur was removed and cleaned of excess tissue and wrapped in saline soaked gauze and stored at -20°C . The left femur was similarly removed, fixed in 10% neutral buffered formalin, and then stored in 70% ethanol at 4°C until analyzed.

Plasma 25OHD Measurement. Plasma 25OHD₃ and 25OHD₂ were measured by liquid chromatography mass spectrometry (IntelimuneWest Lafayette, IN). d_6 -25OHVD₃ and d_6 -25OHVD₂ (Medical Isotopes, Pelham, NH) were used as internal standards. Rat plasma samples were spiked with the internal standards and then extraction by methyl-*tert*-butyl ether (MTBE). The solvent was evaporated, and the remaining residue was derivatized/reconstituted with Secosteroid Signal Enhancing Tag (SecoSET) derivatization reagents (Intelimune, West Lafayette, IN). The derivatized samples were analyzed using multiple reaction monitoring using an Applied Biosystems MDS SCIEX 4000 QTRAP (Applied Biosystems, Carlsbad, CA) interfaced with an automated Shimadzu HPLC (Shimadzu Scientific Instruments, Columbia, MD). Certified vitamin D human serum standards (NIST standard reference material 972) were also assayed as a quality control measure. The assayed concentration of 25OHD₃ and 25OHD₂ in the standard was within 2.6% and 4.5% of the NIST specified value, respectively. The limit of quantification for this method was 0.025 nmol/L for 25OHD₃ and 0.024 nmol/L for 25OHD₂.

Bone Measurements and Mechanical Properties. Right femurs were thawed and hydrated in 0.9% saline for 36 h. Length and width at the midpoint were measured using digital Vernier calipers. Bone density was determined by underwater weighing using an analytical balance (model AG204, Mettler Toledo, Columbus, OH). Femoral breaking strength at the midshaft was assessed with a shear test using an MTS Sintech 10 (MTS, Eden Prairie, MN).^{15,16}

Peripheral Quantitative Computed Tomography (pQCT). Left femurs from animals fed the lowest and highest doses of vitamin D were analyzed by pQCT (Stratec Electronics). Femurs were scanned in two 1 mm slices at 18 and 50% from the distal end of the bone. The 18% site was chosen as a site that would be rich in trabecular bone while avoiding the condyles. The 50% site represents a cortical rich region.¹⁶ Scans were performed with a 0.07 mm voxel size. Thresholds for the segmentation of trabecular and cortical

bone were set at 500 mg/cm³ and 900 mg/cm³. Parameters evaluated by pQCT included total, cortical, and trabecular bone mineral content (BMC), BMD, and area, cortical thickness, endosteal circumference, and periosteal circumference.

Microcomputed Tomography. The same bones that were analyzed by pQCT were also analyzed by microCT with a μCT 40 scanner (Scanco Medical, Switzerland). The trabecular bone at the distal femur was scanned at an isotropic resolution of 16 μm . The bones were scanned in 62 contiguous slices beginning at 18% from the distal end to match the pQCT measurement site and proceeded distally covering a distance of 1 mm. Trabecular bone was delimited by manually contouring the region of interest for every 10th slice and using the automated morph program to interpolate shape changes between the contoured slices. Segmentation values were sigma = 0.8 and support = 1, and a binarization threshold of 313 was visually chosen. All trabecular bone parameters were calculated using a 3D direct model. Parameters evaluated for trabecular bone included tissue volume (TV, mm³), bone volume (BV, mm³), relative bone volume (BV/TV, %), connectivity density (Conn.D, 1/mm³), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm), and trabecular separation (Tb.Sp, mm). Cortical bone parameters were assessed at the midshaft as previously described.¹⁶ Briefly, a total of 50 slices were acquired at a 16- μm resolution. Cortical bone was separated using a semiautomatic contour tracking algorithm to detect the outer and inner boundaries of the cortex. Parameters evaluated for cortical bone included bone volume, cortical thickness (mm), and polar moment of inertia (pMOI, mm⁴).

Statistical Analysis. Statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, NC) using two-way ANOVA. For all analyses, significance was accepted at $p < 0.05$.

RESULTS

Body Weight and Food Intake. There were no differences in body weight or food intake between any of the groups.

Dietary Vitamin D Content. The analyzed vitamin D contents and target values of the experimental diets are given in Table 1.

Plasma 25OHD Status. Plasma 25OHD increased with increasing doses of vitamin D from both crystalline vitamin D₃ and vitamin D₂-rich yeast baked into bread. The relationship between dietary vitamin D and plasma 25OHD was curvilinear (Figure 1) for each vitamin D source. However, rats fed vitamin D₂-rich yeast achieved lower levels of plasma 25OHD than rats fed vitamin D₃. The response of plasma 25OHD to vitamin D₃ was more than twice that of vitamin D₂-rich yeast.

Bone Measurements. Bone length, width, density, and peak breaking strength did not differ by vitamin D dose or source (Table 2).

Results from pQCT showed that vitamin D dose had a significant effect on trabecular BMC ($p = 0.002$), BMD ($p = 0.004$), and area ($p = 0.049$) at the distal femur. There was no significant effect of vitamin D source, nor an interaction of dose and source, on either of these parameters. Between rats fed the lowest and highest doses of vitamin D₂ yeast, there was a 12.5% increase in trabecular BMC, a 23.1% increase in trabecular BMD, and an 8.8% increase in trabecular area. Between rats fed the lowest and highest doses of vitamin D₃, there was a 1.3% increase in trabecular BMC, an 8.0% increase in trabecular BMD, and a 6.1% increase in trabecular area. Other parameters at the distal femur did not differ due to vitamin D dose or source (Table 3). At the midshaft, we did not observe any differences in BMC, BMD, cortical thickness, or cortical area. However, vitamin D dose had a small but statistically significant effect on periosteal

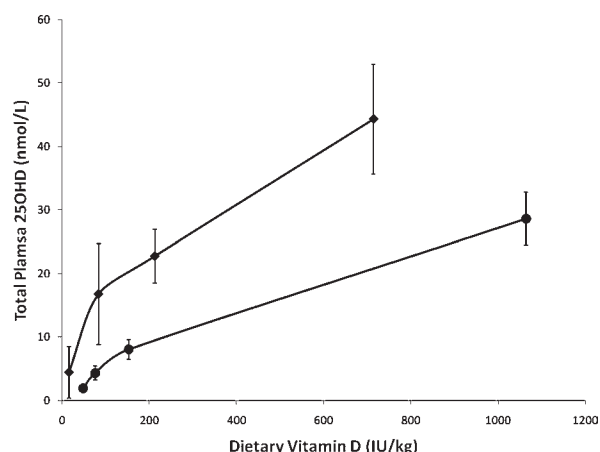


Figure 1. Vitamin D dose—response in rats fed vitamin D₃ (diamonds) or vitamin D₂-rich yeast incorporated into bread (circles) for 8 weeks ($n = 10/\text{diet}$). Error bars represent standard deviation. ANOVA p -values: vitamin D source, <0.0001; dose within source, <0.0001.

Table 1. Vitamin D Content of Diets

target level (IU/kg diet)	analytical level (IU/kg)	
	vitamin D ₂	vitamin D ₃
25 D ₃	ND ^a	16
100 D ₃	ND	84
200 D ₃	ND	213
1000 D ₃	ND	740
25 D ₂ Bread	49	ND
100 D ₂ Bread	76	ND
200 D ₂ Bread	153	ND
1000 D ₂ Bread	1064	ND

^a ND = not detected. Vitamin D₂ and vitamin D₃ were measured using HPLC in the laboratory of Dr. Michael Holick.¹⁴

circumference ($p = 0.03$), endosteal circumference ($p = 0.01$), and total area ($p = 0.03$) at the midshaft. There was no effect of vitamin D source, nor an interaction of vitamin D dose and source, on these measurements. Between rats fed the lowest and highest doses of vitamin D₂ yeast, there was a 2.1% increase in periosteal circumference, a 3.8% increase in endosteal circumference, and a 4.3% increase in total area. Between rats fed the lowest and highest doses of vitamin D₃, there was a 1.8% increase in periosteal circumference, a 1.8% increase in endosteal circumference, and a 3.5% increase in total area.

MicroCT analysis revealed that, at the distal site, vitamin D dose had a significant effect on trabecular bone volume ($p = 0.02$) and connectivity density ($p = 0.03$). There was no effect of vitamin D source, nor an interaction of dose and source, on either parameter. Between rats fed the lowest and highest levels of vitamin D₂ yeast, there was a 26.2% increase in trabecular bone volume and a 22.6% increase in connectivity density. Between rats fed the lowest and highest levels of vitamin D₃, there was a 2.7% increase in trabecular bone volume and a 3.5% increase in connectivity density. No differences were seen in trabecular number, trabecular separation, or trabecular thickness. MicroCT measurements at the midshaft were not significantly different due to vitamin D source or dose (Table 3).

DISCUSSION

Vitamin D-rich foods currently available in the market are not sufficient for most Americans to meet their daily vitamin D requirements. We have demonstrated here that consumption of vitamin D₂-rich yeast incorporated into bread results in increased plasma 25OHD status and improved bone quality in growing, vitamin D-deficient rats. This suggests that bread vitamin D made with high vitamin D yeast could be a valuable new source of vitamin D in the diet. Because vitamin D₂ is naturally produced by yeast in response to UV light, this bread represents a novel, naturally rich food source of vitamin D.

Bread fortified with vitamin D₃ has previously been investigated for its ability to raise vitamin D status. Elderly subjects who were given a daily serving of bread fortified with 5000 IU vitamin D₃ for one year experienced an average increase in serum 25OHD of 98 nmol/L.¹⁷ In healthy adult women randomized to consume wheat or rye bread fortified with vitamin D₃ at a level of 400 IU/day for three weeks, serum 25OHD increased by 16.3 and 14.9 nmol/L, respectively, neither of which was significantly different than the increase from an equivalent dose of a vitamin D supplement.¹⁸ We fed bread made with yeast containing high levels of vitamin D₂ to vitamin D-deficient rats at four doses and observed an increase in plasma 25OHD status with increasing vitamin D₂ intake. These data confirm that vitamin D₂ from bread made from UV-irradiated, vitamin D₂-rich yeast is bioavailable and represents a potential new food source of vitamin D. In the United States, bread may be fortified with vitamin D at levels up to 90 IU/100 g, the maximum level allowed for grain products.⁴ Fortifying bread at this level could provide Americans, who eat on average 79 g of bread per day,¹² with an additional 71.1 IU of vitamin D per day. If the fortification allowance for bread was increased, bread made with vitamin D₂-rich yeast could provide even more vitamin D.

Previously, the dose—response relationship between dietary vitamin D₃ and plasma 25OHD has been described as curvilinear, with plasma 25OHD increasing rapidly at low dietary vitamin D₃ levels and approaching a plateau at higher dietary vitamin D₃ levels.¹⁹ In rats and mice, Fleet et al.²⁰ found a curvilinear relationship between dietary vitamin D₃ and 25OHD with an inflection point around 200 IU/kg diet. Similarly, our data demonstrate a curvilinear relationship between vitamin D intake and plasma 25OHD for both vitamin D₃ and vitamin D₂-rich yeast. For both vitamin D sources, we observed a steep increase in plasma 25OHD with increasing dietary vitamin D below ~100 IU/kg diet and a more shallow increase in plasma 25OHD at higher doses. The inflection point appears to be between 100 and 200 IU/kg. Though the shape of the curve was similar for both vitamin D sources, plasma 25OHD levels achieved were lower in rats fed vitamin D₂-rich yeast compared to that in rats fed vitamin D₃. This difference in plasma 25OHD could represent differences in bioavailability or differences in clearance rate between the two forms.

There has been much debate as to whether vitamin D₂ and D₃ are equivalent in their ability to raise vitamin D status. Adult males given a single large dose of 50,000 IU of either vitamin D₂ or D₃ had similar initial rises in serum 25OHD.²¹ However, after three days, serum 25OHD in the subjects given vitamin D₂ began to decline, while serum 25OHD remained elevated in the subjects given vitamin D₃. Similarly, in elderly women, a single oral dose of 300,000 IU vitamin D₃ caused a greater initial rise and a slower rate of decline in serum 25OHD, compared with

Table 2. Body Weight and Femur Characteristics of Rats Fed Varying Levels of Vitamin D3 or Vitamin D2-Rich Yeast Baked into Bread for 8 Weeks

	vitamin D3				vitamin D2-rich yeast bread			
	25 IU	100 IU	200 IU	1000 IU	25 IU	100 IU	200 IU	1000 IU
final body weight, g	427.8 ± 32.7 ^a	452.9 ± 28.7	431.6 ± 28.9	445.4 ± 42.6	429.0 ± 23.8	432.2 ± 35.1	442.2 ± 30.2	438.9 ± 33.1
femur								
length (mm)	39.82 ± 0.73	40.03 ± 0.76	39.38 ± 0.87	39.89 ± 0.75	39.64 ± 0.77	39.52 ± 1.05	39.75 ± 0.47	39.86 ± 1.00
width m/l (mm)	4.33 ± 0.19	4.51 ± 0.22	4.35 ± 0.21	4.31 ± 0.16	4.28 ± 0.15	4.33 ± 0.21	4.42 ± 0.14	4.36 ± 0.19
width a/p (mm)	3.57 ± 0.09	3.70 ± 0.12	3.59 ± 0.19	3.69 ± 0.16	3.60 ± 0.14	3.61 ± 0.19	3.64 ± 0.11	3.71 ± 0.22
density (g/cm ³)	1.309 ± 0.023	1.385 ± 0.023	1.392 ± 0.020	1.407 ± 0.022	1.396 ± 0.020	1.408 ± 0.029	1.400 ± 0.017	1.400 ± 0.012
peak breaking strength (lb)	59.90 ± 6.56	56.92 ± 7.53	57.68 ± 7.07	53.20 ± 6.46	55.55 ± 9.11	54.85 ± 6.96	58.34 ± 5.89	58.89 ± 7.65

^a Values are the mean ± SD. There were no statistically significant differences due to vitamin D dose or source for any measurements.

Table 3. Femur pQCT and MicroCT Characteristics of 11 Wk-Old Vitamin D-Deficient Rats Fed Varying Levels of Vitamin D3 or Vitamin D2-Rich Yeast Baked into Bread for 8 Weeks^a

	vitamin D3		vitamin D2-rich yeast bread		ANOVA
	low	high	low	high	dose <i>p</i> -value
Distal pQCT					
total BMD (mg/cm ³)	556.07 ± 33.8 ^b	544.10 ± 32.3	554.08 ± 18.8	552.40 ± 22.7	NS ^c
total BMC (mg/mm)	11.85 ± 0.91	11.43 ± 0.70	11.09 ± 0.44	11.92 ± 0.75	NS
trabecular BMD (mg/cm ³)	249.68 ± 24.5	253.05 ± 19.9	245.5 ± 15.2	276.15 ± 30.7	0.027
trabecular BMC (mg/mm)	3.62 ± 0.57	3.91 ± 0.68	3.42 ± 0.31	4.21 ± 0.82	0.010
total area (mm ²)	21.37 ± 1.98	22.16 ± 2.05	20.50 ± 0.99	21.63 ± 1.85	NS
trabecular area (mm ²)	14.50 ± 1.69	15.38 ± 2.03	13.91 ± 0.93	15.14 ± 1.80	0.049
Distal MicroCT					
TV (mm ³)	12.21 ± 1.21	12.63 ± 1.19	11.77 ± 0.81	12.68 ± 1.45	NS
BV (mm ³)	1.83 ± 0.34	1.88 ± 0.25	1.72 ± 0.25	2.17 ± 0.49	0.030
BV/TV (%)	0.150 ± 0.204	0.149 ± 0.013	0.146 ± 0.018	0.170 ± 0.027	NS
Conn.D (1/mm ³)	42.3 ± 6.7	43.8 ± 7.7	41.6 ± 6.7	51.0 ± 8.7	0.027
Tb.N (1/mm)	2.92 ± 0.24	2.94 ± 0.26	2.84 ± 0.20	3.02 ± 0.22	NS
Tb.Th (mm)	0.0825 ± 0.0048	0.0820 ± 0.0051	0.0821 ± 0.0034	0.0815 ± 0.0037	NS
Tb.Sp (mm)	0.3435 ± 0.034	0.3462 ± 0.033	0.3639 ± 0.026	0.3343 ± 0.032	NS
Midshaft pQCT					
total BMD (mg/cm ³)	898.31 ± 34.4	879.10 ± 26.2	892.16 ± 27.8	874.30 ± 33.3	NS
total BMC (mg/mm)	11.15 ± 0.76	11.30 ± 0.57	11.00 ± 0.58	11.23 ± 0.57	NS
cortical BMD (mg/cm ³)	1416.5 ± 11.3	1417.7 ± 9.8	1413.3 ± 9.5	1415.6 ± 12.1	NS
cortical BMC (mg/mm)	10.51 ± 0.74	10.66 ± 0.53	10.37 ± 0.58	10.58 ± 0.52	NS
total area (mm ²)	12.41 ± 0.61	12.85 ± 0.62	12.33 ± 0.53	12.86 ± 0.91	0.031
cortical area (mm ²)	7.41 ± 0.48	7.52 ± 0.34	7.33 ± 0.41	7.47 ± 0.38	NS
cortical thickness (mm)	0.727 ± 0.041	0.720 ± 0.024	0.720 ± 0.036	0.714 ± 0.018	NS
periosteal circ (mm)	12.49 ± 0.31	12.71 ± 0.31	12.45 ± 0.27	12.71 ± 0.45	0.032
endosteal circ (mm)	7.92 ± 0.27	8.19 ± 0.30	7.92 ± 0.28	8.22 ± 0.45	0.011
Midshaft MicroCT					
TV (mm ³)	5.87 ± 0.38	5.94 ± 0.27	5.79 ± 0.30	5.92 ± 0.29	NS
BV (mm ³)	5.56 ± 0.37	5.62 ± 0.27	5.48 ± 0.29	5.60 ± 0.27	NS
BV/TV (%)	0.9468 ± 0.0024	0.9467 ± 0.0021	0.9463 ± 0.0019	0.9459 ± 0.0019	NS
cortical thickness (mm)	0.686 ± 0.032	0.681 ± 0.025	0.680 ± 0.031	0.676 ± 0.023	NS
pMOI (mm ⁴)	20.15 ± 2.2	21.18 ± 2.1	19.70 ± 1.7	21.22 ± 2.6	NS

^a The distal and midshaft sites were at 18 and 50% from the distal end, respectively. ^b Values are the mean ± SD. There were no significant effects of source or dose × source interaction for any parameters. ^c NS, not significant.

that in women dosed with vitamin D₂.²² In contrast, daily dosing for 11 weeks with 1000 IU of vitamin D₂ or D₃, as either a capsule²³ or as fortified orange juice,²⁴ produced and maintained similar increases in serum 25OHD in healthy adults. Together, these data suggest that vitamin D₂ may indeed have a more rapid turnover rate in the serum than vitamin D₃ but that the difference may be inconsequential when vitamin D is given daily. Contrarily, we found that rats fed vitamin D₂ for eight weeks had lower plasma 25OHD levels than rats fed vitamin D₃, suggesting that in growing rats, vitamin D₂ may have a lower capacity to increase and sustain plasma 25OHD levels than vitamin D₃.

In addition to comparing the effects of vitamin D sources and doses on serum 25OHD status, it is important to also consider whether vitamin D source and dose also have a functional impact. We compared the measurements of bone strength, density, and microarchitecture as functional indicators of vitamin D status. To our knowledge, we are the first to compare the effects of dietary vitamin D₃ and D₂ on bone microarchitecture. Vitamin D is traditionally believed to influence bone quality after renal conversion to the endocrine hormone 1,25-dihydroxyvitamin D (1,25(OH)₂D) through its role as a regulator of calcium absorption and PTH production,¹ and it may also have direct effects on bone through local conversion to 1,25(OH)₂D within bone cells.²⁵ Rickets is associated with very low levels of circulating 25OHD (typically less than 20 nmol/L).²⁶ Fleet et al.²⁰ showed that mice fed a diet containing 25 IU/kg vitamin D₃ from weaning to 14 weeks of age attained borderline deficient serum 25OHD levels (25–40 nmol/L) and reduced serum 1,25-(OH)₂D levels, and had significantly reduced femoral BMD and BMC compared to that in mice fed 1000 IU/kg vitamin D₃. Anderson et al.²⁷ compared bone histomorphometric measures among rats who received daily vitamin D₃ doses ranging from 50 to 500 ng/day (approximately 100–1000 IU/kg diet) from 10 to 30 weeks of age. At the distal femur, they observed increased bone volume fraction, trabecular number, and decreased trabecular separation between rats fed 500 ng/day vitamin D₃ and rats fed 50 ng/day. We found a similar positive effect of high vitamin D intake in the growing rat on trabecular bone BMD, BMC, area, bone volume, and connectivity density at the distal femur. The effect of vitamin D dose did not differ between crystalline vitamin D₃ and vitamin D₂-rich yeast, indicating that the two sources have equivalent ability to improve trabecular bone. We also found that higher vitamin D intake increased endosteal circumference, periosteal circumference, and total cross-sectional area of the femur midshaft. This effect did not differ due to vitamin D source, again suggesting that crystalline vitamin D₃ and vitamin D₂-rich yeast are equivalent in their effect on cortical geometry.

There are some limitations to our work. First, we did not evaluate the bioavailability of vitamin D₂-rich yeast in bread compared to that of crystalline vitamin D₂. However, since many food and supplement sources of vitamin D contain vitamin D₃, we felt that vitamin D₃ was an appropriate comparison. Also, we did not incorporate the crystalline vitamin D₃ into bread before adding it to the rat diet. Adding vitamin D₃ directly to the diet is more representative of the range of vitamin D fortified foods available on the market; however, since our vitamin D₃ diets lacked the bread matrix of the vitamin D₂ diets, we cannot be certain that any differences between the diets were due to the vitamin D form and not other components of the bread. Second, our experiment was performed in young, growing, vitamin D-deficient rats. As a result, our findings may not apply to other life stages or to vitamin D-replete individuals. Third, measuring tissue distribution of the

vitaminers and their metabolites would have been useful to fully assess the bioefficacy and metabolism of these two vitamin D sources.

In summary, we have demonstrated vitamin D from vitamin D₂-rich yeast is bioavailable from bread. When bread made with this yeast was fed to growing, vitamin D-deficient rats, plasma 25OHD increased in a dose-dependent manner. Though plasma 25OHD levels were lower in rats fed bread made with vitamin D₂-rich yeast than in rats fed vitamin D₃, the two sources were equally effective in improving trabecular BMC, BMD, bone volume, and connectivity density, and cortical geometry. Therefore, bread made with vitamin D₂-rich yeast could be used as a new food source of vitamin D.

AUTHOR INFORMATION

Corresponding Author

*Phone: (765) 494-8237. Fax: (765) 494-0674. E-mail: weavercm@purdue.edu.

Funding Sources

This work was supported by Lallemand/American Yeast.

ABBREVIATIONS USED

1,25OH₂D, 1,25-dihydroxyvitamin D; 25OHD, 25-hydroxyvitamin D; AI, adequate intake; BMC, bone mineral content; BMD, bone mineral density; BV, bone volume; Conn.D, connectivity density; IU, international units; microCT, microcomputed tomography; pMOI, polar moment of inertia; PTH, parathyroid hormone; pQCT, peripheral quantitative computed tomography; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TV, tissue volume.

REFERENCES

- (1) Xue, Y.; Fleet, J. C. Intestinal vitamin D receptor is required for normal calcium and bone metabolism in mice. *Gastroenterol.* **2009**, *136*, 1317–1327.
- (2) Holick, M. F. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. *Am. J. Clin. Nutr.* **2004**, *80*, 1678S–1688S.
- (3) Ginde, A.; Liu, M.; Camargo, C. J. Demographic differences and trends of vitamin D insufficiency in the US population, 1988–2004. *Arch. Intern. Med.* **2009**, *169*, 626–632.
- (4) Calvo, M.; Whiting, S.; Barton, C. Vitamin D fortification in the United States and Canada: current status and data needs. *Am. J. Clin. Nutr.* **2004**, *80*, 1710S–1716S.
- (5) Holick, M.; Shao, Q.; Liu, W.; Chen, T. The vitamin D content of fortified milk and infant formula. *N. Engl. J. Med.* **1992**, *326*, 1178–1181.
- (6) Bailey, R.; Dodd, K.; Goldman, J.; Gahche, J.; Dwyer, J.; Moshfegh, A.; Sempas, C.; Picciano, M. Estimation of total usual calcium and vitamin D intakes in the United States. *J. Nutr.* **2010**, *140*, 817–822.
- (7) Institute of Medicine (IOM). *Dietary Reference Intakes for Calcium and Vitamin D*; The National Academies Press: Washington, DC, 2011.
- (8) Patterson, K. Y.; Phillips, K. M.; Horst, R. L.; Byrdwell, W. C.; Exler, J.; Lemar, L. E.; Holden, J. M. Vitamin D content and variability in fluid milks from a US Department of Agriculture nationwide sampling to update values in the National Nutrient Database for Standard Reference. *J. Dairy Sci.* **2010**, *93*, 5082–5090.
- (9) Dietary Guidelines Advisory Committee. *Report of the Dietary Guidelines Advisory Committee on the dietary guidelines for Americans*, 2005; U.S. Department of Agriculture: Washington, D.C., 2004.
- (10) Weaver, C. M.; Fleet, J. C. Vitamin D requirements: current and future. *Am. J. Clin. Nutr.* **2004**, *80*, 1735S–1739S.

- (11) Jasinghe, V. J.; Perera, C. O.; Barlow, P. J. Bioavailability of vitamin D₂ from irradiated mushrooms: an in vivo study. *Br. J. Nutr.* **2005**, *93*, 951–955.
- (12) Agricultural Research Service. *Continuing Survey of Food Intakes by Individuals 1994–96*; U.S. Department of Agriculture: Beltsville, MD.
- (13) Reeves, P. G.; Nielsen, F. H.; Fahey, G. C., Jr. 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.
- (14) Chen, T.; Chimeh, F.; Lu, Z.; Mathieu, J.; Person, K.; Zhang, A.; Kohn, N.; Martinello, S.; Berkowitz, R.; Holick, M. Factors that influence the cutaneous synthesis and dietary sources of vitamin D. *Arch. Biochem. Biophys.* **2007**, *460*, 213–217.
- (15) American Society of Agricultural and Biological Engineers. S459: Shear and three-point bending test of animal bone. *ASABE Standards*; 2007.
- (16) Weaver, C. M.; Janle, E.; Martin, B.; Browne, S.; Guiden, H.; Lachcik, P.; Lee, W. H. Dairy versus calcium carbonate in promoting peak bone mass and bone maintenance during subsequent calcium deficiency. *J. Bone Miner. Res.* **2009**, *24*, 1411–1419.
- (17) Mocanu, V.; Stitt, P. A.; Costan, A. R.; Voroniuc, O.; Zbranca, E.; Luca, V.; Vieth, R. Long-term effects of giving nursing home residents bread fortified with 125 microg (5000 IU) vitamin D(3) per daily serving. *Am. J. Clin. Nutr.* **2009**, *89*, 1132–1137.
- (18) Natri, A. M.; Salo, P.; Vikstedt, T.; Palssa, A.; Huttunen, M.; Karkkainen, M. U.; Salovaara, H.; Piironen, V.; Jakobsen, J.; Lamberg-Allardt, C. J. Bread fortified with cholecalciferol increases the serum 25-hydroxyvitamin D concentration in women as effectively as a cholecalciferol supplement. *J. Nutr.* **2006**, *136*, 123–127.
- (19) Heaney, R. P.; Davies, K. M.; Chen, T. C.; Holick, M. F.; Barger-Lux, M. J. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *Am. J. Clin. Nutr.* **2003**, *77*, 204–210.
- (20) Fleet, J. C.; Gliniak, C.; Zhang, Z.; Xue, Y.; Smith, K. B.; McCreedy, R.; Adedokun, S. A. 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.
- (21) Armas, L. A.; Hollis, B. W.; Heaney, R. P. Vitamin D₂ is much less effective than vitamin D₃ in humans. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 5387–5391.
- (22) Romagnoli, E.; Mascia, M. L.; Cipriani, C.; Fassino, V.; Mazzei, F.; D'Erasmus, E.; Carnevale, V.; Scillitani, A.; Minisola, S. 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.* **2008**, *93*, 3015–3020.
- (23) Holick, M. F.; Biancuzzo, R. M.; Chen, T. C.; Klein, E. K.; Young, A.; Bibuld, D.; Reitz, R.; Salameh, W.; Ameri, A.; Tannenbaum, A. D. Vitamin D₂ is as effective as vitamin D₃ in maintaining circulating concentrations of 25-hydroxyvitamin D. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 677–681.
- (24) Biancuzzo, R. M.; Young, A.; Bibuld, D.; Cai, M. H.; Winter, M. R.; Klein, E. K.; Ameri, A.; Reitz, R.; Salameh, W.; Chen, T. C.; Holick, M. F. Fortification of orange juice with vitamin D₂ or vitamin D₃ is as effective as an oral supplement in maintaining vitamin D status in adults. *Am. J. Clin. Nutr.* **2010**, *91*, 1621–1626.
- (25) Atkins, G. J.; Anderson, P. H.; Findlay, D. M.; Welldon, K. J.; Vincent, C.; Zannettino, A. C.; O'Loughlin, P. D.; Morris, H. A. Metabolism of vitamin D₃ in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha, 25-dihydroxyvitamin D₃. *Bone* **2007**, *40*, 1517–28.
- (26) Prentice, A. Vitamin D deficiency: a global perspective. *Nutr. Rev.* **2008**, *66*, S153–S164.
- (27) Anderson, P. H.; Sawyer, R. K.; Moore, A. J.; May, B. K.; O'Loughlin, P. D.; Morris, H. A. Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model. *J. Bone Miner. Res.* **2008**, *23*, 1789–97.