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TITLE PAGE

Title: Poor bioavailability of vitamin D_2 from UV-irradiated D_2-rich yeast in rats

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Abbreviations page

Abbreviations

BMC; bone mineral content

BMD; bone mineral density

CV; coefficient of variation

CYP24A1; cytochrome P24A1

DXA; dual-energy X-ray bone densitometry

$D_{2;}$ vitamin D_2

$D_{3;}$ vitamin D_3

LC-MS/MS; liquid chromatography-tandem mass spectrometry

SD; standard deviation

SE; standard error

S-PTH; serum parathyroid hormone

S-Ca; serum calcium

S-Pi; serum phosphate

UV; ultraviolet

1,25$(OH)_2$D; 1,25-dihydroxyvitamin D

24,25$(OH)_2$D; 24,25-dihydroxyvitamin D

25(OH)D; 25-hydroxyvitamin D

Abstract page

Ultraviolet (UV)-irradiated yeast (*Saccharomyces cerevisiae*) can be used to biofortify bakery products with vitamin D, but in bread it was not effective in increasing serum 25-hydroxyvitamin D (25(OH)D) in humans, possibly due to the low digestibility of the yeast matrix. We investigated the effects of vitamin D_2-rich intact yeast cells and their separated fraction, yeast cell walls, which we hypothesized to provide vitamin D_2 in a more bioavailable form, on serum 25(OH)D and its metabolites in growing female Sprague Dawley rats (n=54) compared to vitamin D_2 and D_3 supplements (eight treatment groups: 300 or 600 IU vitamin D/d, and a control group, 8 week intervention). The D_3 supplement groups had the highest 25(OH)D concentrations, and the vitamin D_2 supplement at the 600 IU dose increased 25(OH)D better than any yeast form ($P<0.001$ for all, analysis of covariance, adjusted for body weight). There were no significant differences between the yeast forms at the same dose ($P>0.05$). Serum 24,25-dihydroxyvitamin D ($24,25(OH)_2D$; a vitamin D catabolite) concentrations and the trend in the differences between the groups were in line with 25(OH)D ($P<0.001$ for all). The $24,25(OH)_2D$ to 25(OH)D ratio between the D_2 supplement and the yeast groups did not differ ($P>0.05$). These findings do not support the hypothesis: the ability of the different UV-treated vitamin D_2-containing yeast forms to increase 25(OH)D did not differ and the poor bioavailability of vitamin D_2 in the yeasts compared D_3 or D_2 supplements could not be explained by the increased vitamin D catabolism in the yeast-treated groups.

Keywords: Bioavailability; Biofortification; Rats; UV-irradiated yeast; Vitamin D_2; 25-hydroxyvitamin D

1. Introduction

Due to the limited selection of naturally vitamin D-rich food sources, food fortification with vitamin D has been considered a viable option to improve vitamin D intake in the population [1]. While still debated, vitamin D_3 may be more potent than vitamin D_2 in terms of increasing serum 25-hydroxyvitamin D [25(OH)D, widely regarded as an index of vitamin D status] concentration [2]. Although most vitamin D-fortified foods are enriched with animal-originated vitamin D_3, vitamin D_2 may have other advantages such as its commercial production can be very cost-effective and it is suitable also for use in strict vegetarians [3]. For these reasons vitamin D_2 has potential for broader utilization in food fortification.

In addition to the traditional mode of fortification, in which vitamin D is exogenously added to the product, the vitamin D contents of the foodstuffs can be increased by biofortification means [4]. Amongst others, this can include the ultraviolet (UV) B irradiation of fungi i.e. mushrooms or baker's yeast [5]. UV-irradiated yeast, with enhanced vitamin D_2 levels, can be used to enrich bakery products with vitamin D: irradiated yeast cells retain most of their gassing power which makes them ideal for enrichment of yeast leavened products, such as bread [6]. Hohman et al [7] studied a bread made with UV-treated, vitamin D_2-rich yeast in vitamin D-deficient rats, and vitamin D_2 from yeast was shown to be bioavailable, although not as effective in increasing plasma 25(OH)D concentrations as a vitamin D_3 supplement. Later, Itkonen and coworkers [8] investigated the bioavailability of vitamin D_2 from bread baked with UV-treated yeast in an 8-week winter-based randomized controlled trial in young healthy women. Consumption of bread produced with the UV-treated, vitamin D_2-rich yeast did not increase total 25(OH)D concentration, while women given bread made with regular

yeast and either a vitamin D_2 or D_3 supplement (providing the same level of vitamin D as D_2-enriched bread) had significant improvements in total 25(OH)D concentration. Interestingly, Lipkie et al [9] recently utilized their *in vitro* bioaccessibility model, which simulated digestion in the gastrointestinal tract after ingestion of UV-yeast fortified bread, and found that intact yeast cells were present in the digesta of the yeast fortified bread. These findings suggest a low bioaccessibility of vitamin D_2 in this UV-treated yeast, due to the lower digestibility of the yeast matrix. This may explain the findings of low bioavailability of the vitamin D_2 from the bread used in the study of Itkonen et al. [8]. It is worth noting that UV-irradiated D_2-rich yeast is already used in some breads on the market and despite its vitamin D content, could be a poor source of bioavailable vitamin D.

Due to the matrix effects it is possible that the bioavailability of the D_2 in yeast, and thus the ability to increase serum 25(OH)D, may be better if a separate cell wall fraction of the yeast is used. In that case, the matrix is already broken because the yeast cell walls are separated from the intact yeast cells by autolysis or hydrolysis and centrifugation [10]. Therefore, we hypothesize that yeast cell wall fraction due to potentially better bioavailability of its D_2 may be more capable of improving vitamin D status than the intact yeast cells. Accordingly, we investigated the ability of vitamin D_2-rich intact yeast cells (*Saccharomyces cerevisiae*) and yeast cell walls to increase serum 25(OH)D in growing rats compared to vitamin D_2 and D_3 supplements. In addition, to discount the possibility that vitamin D_2 from the UV-treated yeast is metabolized more rapidly than supplemental vitamin D_2, the concentration of the 24-pathway degradation metabolite, 24,25-dihydroxyvitamin D (24,25$(OH)_2$D) was also assessed. The effects of the different forms of vitamin D on bone development within the growing rats were examined by measuring femoral bone mineral content (BMC), bone area,

and bone mineral density (BMD). Finally, additional information about vitamin D-related metabolism and safety of the different vitamin D sources was obtained by measuring calcium metabolism biomarkers in serum.

2. Methods and materials

2.1 Ethical approval

The project was approved by the Animal Experiment Board in Finland (Laboratory Centre of the University of Helsinki permit number KEK15-009).

2.2 Animals and feeds

Three-week old, Sprague Dawley® Outbred female rats (n=54) were obtained from Harlan Laboratories (Madison, WI, USA). They were placed in the Laboratory Animal Centre of the University of Helsinki and cared for by its personnel. The light/dark cycle was 12 hours and water was provided *ad libitum*. The animals were allowed to acclimatize one week prior the study. During this acclimatization period, the rats were fed with Teklad global 16% protein rodent diet (2916C-031015MA, Harlan Laboratories, Madison, WI, USA). For the experimental period, the rats were stratified into nine groups of six animals each. All diets

were obtained from Harlan laboratories (Madison, WI, USA). The basis of each diet was the AIN-93G diet (recommended for growing rodents) with vitamin-free tested casein (TD. 07669). The diets of the nine groups varied by the amount and source of vitamin D: control diet and diets containing one of two different doses of vitamin D (300 IU/d or 600 IU/d) and as either vitamin D_3 supplement, vitamin D_2 supplement, vitamin D_2-rich intact yeast cells or vitamin D_2-rich yeast cell walls. The diets are described in detail in Table 1. The maximum food consumption was estimated to be 20 grams per day which was provided daily, and the excess food was weighted after the experiment.

Supplemental vitamin D_3 was provided by Harlan Laboratories, whereas supplemental vitamin D_2 was obtained from Sigma-Aldrich (Ergocalciferol, 40 000 000 USP units/g, E5750, CAS 50-14-6, EC 200-014-9, St. Louis, MO, USA). The yeasts were provided by LALLEMAND Inc. (Montreal, Canada). The production process for the UV-irradiated D_2-rich yeast preparations is described elsewhere [6,10]. The D_2 supplements and the yeasts preparations were shipped to Harlan Laboratories where the diets were produced. The exact vitamin D contents of LALLEMAND yeasts were analyzed by Covance (Princeton, NJ, USA) by high-performance liquid chromatography [11]. The amount of D_3 in the D_2 diets was below the detection limit of 20 μg/100g. The vitamin D_2 contents of the spray-dried intact yeast cells and yeast cell walls were 39,250 μg/100 g (1,570,000 IU) and 138,500 μg/100 g (5,540,000 IU), respectively.

2.3 Experiments

The experimental period was for eight weeks during which the rats were allowed to feed *ad libitum*. Each rat was weighted every two weeks (i.e., at 0, 2, 4, 6 and 8 weeks) within the experimental period, when the rats were 4, 6, 8, 10 and 12 weeks of age, respectively. After eight weeks, the rats were euthanized with CO_2 and their necks were broken. Blood was drawn by cardiac puncture and centrifuged soon after drawing. The serum samples were stored at -70°C until required for analyses.

2.4 Biochemical analyses

Serum 25(OH)D_3, 25(OH)D_2, 24,25$(OH)_2D_3$ and 24,25$(OH)_2D_2$ concentrations were analyzed by the *Cork Centre for Vitamin D and Nutrition Research* at University College Cork in Ireland using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, modified from a validated method for the analysis of 25(OH)D_3, 25(OH)D_2, and 24,25$(OH)_2D_3$ [12]. Total 25(OH)D and 24,25$(OH)_2$D concentrations were calculated as [25(OH)D_3 + 25(OH)D_2], and [24,25$(OH)_2D_3$ + 24,25$(OH)_2D_2$], respectively. The quantification range (nmol/L) was 2.52-323 for 25(OH)D_3, 1.31-168 for 25(OH)D_2, 0.72-185 for 24,25(OH)D_3, and 0.69-176 for 24,25(OH)D_2. The CV (%) for all metabolites was <10%. Further details of the serum extraction and LC-MS/MS analysis can be found in the Supplementary material.

Serum parathyroid hormone (S-PTH) concentrations were analyzed by enzyme-labeled immunometric assay with the Rat Intact PTH ELISA Kit (Immutopics Inc., San Clemente, CA, USA) at the Department of Food and Nutrition, University of Helsinki [13]. Due to the heterogeneity of the S-PTH results, the analyses were carried out in duplicate, however, the results were consistent. Serum calcium (S-Ca) and serum phosphate (S-Pi) concentrations were analyzed by a spectrophotometric method by Konelab20 automatic analyser (Thermo Clinical Labsystems Oy, Espoo, Finland) at the Department of Food and Nutrition, University of Helsinki [14,15]. The inter- and intra-assay CV (%) for S-Ca and S-Pi were <4.6% and <4.6%, respectively.

2.5 Bone measurements (femoral bone area, BMC and BMD)

Left hind legs were excised within a few hours following kill, using scissors and scalpels. Immediately after removal, femoral bone area (cm^2), BMC (g) and BMD (g/cm^2) were measured with a dual-energy X-ray bone densitometry device (DXA) (Lunar Piximus, GE Healthcare, Little Chalfont, UK) [16]. Because the whole leg was measured, the region of interest needed to be adjusted manually to cover only the femur. Prior to measurement, a quality control check was performed using a phantom mouse as per DXA supplier instructions.

2.6 Statistical analyses

The choice of number of animals per treatment group in the present study was informed by data on the average number of animals used in cognate vitamin D feeding studies in rats [17,18]. In addition, *post hoc* sample size calculations showed that the number of rats per group was sufficient to show significant differences (at an alpha of 0.05 and 80% power) in serum total 25(OH)D, as the priority outcome measure, between the different vitamin D forms when compared at a similar dose level.

Data are presented in tables as means ± standard deviation (SD), and in figures as means ± standard error (SE). The normality and homogeneity of the data were verified and log-transformed to improve normality, when needed. All tests were considered significant at $P<0.05$. Differences between the treatment groups were studied by one-way analysis of variance and analysis of covariance using rat body weight at the end of the intervention as a covariate. *Post hoc* tests for the differences between the groups were carried out with Fisher's least significant difference test. Spearman correlation coefficients were used to test for significant associations between vitamin D metabolites and bone measurements for all rats, and separately according to the vitamin D source (D_3 or D_2). Statistical analysis was performed by SPSS Statistics version 23 (IBM, Armonk, NY, USA).

3. Results

3.1 Food and vitamin D intake

Descriptive statistics of the vitamin D intake and rat body weight are described in the Table 2. When excess food was weighted after the experiment, the actual daily vitamin D intake was lower than excepted (Table 2).

3.2 Serum 25(OH)D concentrations

There was no trace of $25(OH)D_2$ in serum of animals fed supplemental vitamin D_3, nor $25(OH)D_3$ in serum of animals fed supplemental vitamin D_2 (data not shown). Statistically significant differences in serum total 25(OH)D concentration were found between the study groups ($P<0.001$, Figure 1A). Serum total 25(OH)D in the control group was lower than in all other groups ($P<0.001$-0.005), with the exception of the lower dose intact yeast cell group ($P>0.05$). The higher vitamin D_3 dose group differed from all other groups ($P<0.001$), whereas the lower vitamin D_3 dose group differed from all other groups ($P<0.001$-0.002), except from the higher dose vitamin D_2 group ($P>0.05$): vitamin D_3 supplement-fed groups had the highest serum total 25(OH)D concentrations post intervention. At the lower dose level (300 IU/d), feeding the vitamin D_2 supplement resulted in higher serum total 25(OH)D than feeding with intact yeast cells ($P=0.026$), but no differences compared to cell wall were evident ($P>0.05$). However, at the higher dose level (600 IU/d), the vitamin D_2 group had a higher serum total 25(OH)D than either of the yeast forms ($P=0.002$-0.020). There were

statistical differences evident between the intact yeast cell and cell wall fraction groups when compared at the same dose levels (P>0.05).

3.3 Serum 24,25(OH)$_2$D concentrations and 24,25(OH)$_2$D to 25(OH)D ratio

Serum 24,25(OH)$_2$D correlated well with total 25(OH)D (Supplemental Table). Differences in serum 24,25(OH)$_2$D and 24,25(OH)$_2$D to 25(OH)D ratio were significant between the groups (P for both <0.001) (Figures 1B, 1C). All treatment groups had higher serum 24,25(OH)$_2$D concentrations than the control group (P<0.001-0.015), and higher doses of each vitamin D form resulted in higher serum 24,25(OH)$_2$D concentrations compared to the lower dose (P=0.001-0.041). Highest serum concentrations were present in higher dose vitamin D_3 group (P<0.001-0.037) while vitamin D_2 and cell wall groups were on similar level and did not differ from each other (P>0.05). The intact yeast cell groups had the lowest serum 24,25(OH)$_2$D concentrations: the lower dose group differed significantly from all other groups (P<0.001-0.039), except from the lower dose D_2 supplement group (P>0.05), and the higher dose differed from all other groups (P<0.001-0.016), except from the cell wall groups (P>0.05). Concerning the 24,25(OH)$_2$D to 25(OH)D ratio, with the exception of the high dose vitamin D_3 (0.79; P>0.05), all other groups had a higher ratio than control (0.69) (P<0.001-0.002). Higher dose D_3 differed from the other treatment groups (P<0.001-0.033). The ratios between other groups or doses did not differ from each other (P>0.05) and ranged from 1.00 to 1.14.

3.4 Growth and femoral bone development

There were no significant differences in mean body weights between the groups at the end of the intervention (Table 2). There were no significant correlations between bone parameters and serum total 25(OH)D (Supplemental Table), but bone area and BMC correlated negatively with 24,25$(OH)_2$D to 25(OH)D ratio when all rats were pooled, and a negative correlation between bone area and 24,25$(OH)_2$D to 25(OH)D among vitamin D_2 treated rats was also evident. In bone area and BMC tended to be differences between the treatment groups (P=0.068 and P=0.069, respectively) (Figures 1D, 1E). The adjusted mean values of bone area and BMC tended to be highest in the higher dose vitamin D_3 group followed by the lower dose vitamin D_3 and higher dose cell wall groups. There were no significant differences evident in mean BMD values (P=0.127) (Figure 1F).

3.5 Calcium metabolism biomarkers

S-PTH correlated positively with 25(OH)D and 24,25$(OH)_2$D when all rats were pooled. In vitamin D_3 treated rats significant positive correlations between S-PTH and 24,25$(OH)_2$D, BMC and bone area were evident, but not among vitamin D_2 treated rats. Significant differences in S-Pi and S-PTH concentrations between the high vitamin D_3 dose and all other groups were seen (P=0.001 for both), but no differences between intact yeast cell and cell wall groups were present for either marker (Figures 1G, 1H). However, results from S-PTH assay showed great deviation within some groups. There were four animals with S-PTH

concentration higher than 2000 pg/mL, three of which belong to group fed with higher vitamin D_3 supplement dose and the remaining one to group fed with smaller vitamin D_3 dose. In the other groups, the variation was smaller. No differences in S-Ca concentration between the groups were present (P=0.640, Figure 1I).

4. Discussion

In the present study of growing female Sprague Dawley rats, the vitamin D bioavailability and safety of UV-irradiated vitamin D_2-rich intact yeast cells and their cell wall fraction in different doses were examined. Calcium metabolism biomarker data in the present study suggested both yeast forms were seen to be safe. The study showed that while both vitamin D_2 and vitamin D_3 were capable in increasing serum total 25(OH)D, all vitamin D_2-diets showed lower effectiveness in terms of raising serum total 25(OH)D compared to supplemental vitamin D_3, at both dose levels. These vitamer-specific findings in young rats are very much in keeping with recent studies in humans. For example, Tripkovic et al. [2] in their systematic review showed that vitamin D_3 was more effective in increasing 25(OH)D concentrations than vitamin D_2.

In terms of bioavailability, vitamin D_2-rich yeast used in the present study as intact yeast cells (supplying vitamin D at the 300 IU dose level) and yeast cell walls (at 300 and 600 IU dose levels) were effective in increasing serum total 25(OH)D compared to control, even if for the most part were less effective than the vitamin D_2 supplement. Similarly, Hohman et al. [7] in

their earlier study of vitamin D-deficient rats, observed that vitamin D_2 from UV-irradiated yeast was bioavailable, but not as effective at increasing plasma 25(OH)D concentration as vitamin D_3. It should be noted, however, they did not use a vitamin D_2 supplement as a positive control. Furthermore, Hohman and coworkers used vitamin D-deprived animals in their study [7]. This may be of consequence, as it has been seen in human studies that individuals with the lowest baseline 25(OH)D concentrations have the strongest response to increased vitamin D intake [19]. The estimated levels of ingested vitamin D in the present study, even though the dietary levels were lower than excepted, were approximately 20-fold higher than the recommended daily intake for growing rats [17,18]. Thus, the rats were of adequate vitamin D status. Of note, in their 8-week, winter-based intervention study of young healthy women in Finland, Itkonen et al [8] showed that consumption of bread produced with the UVB-treated, vitamin D_2-rich intact yeast failed to lead to an increase in serum total 25(OH)D concentration, even though a significant increase in serum total 25(OH)D was evident in the women receiving bread made with regular yeast and either a vitamin D_2 (or D_3) supplement. Likewise, in our study, the lower dose of intact yeast cells was not able to increase serum total 25(OH)D compared to the control group. Lipkie et al [9] have demonstrated using their *in vitro* digestion model that the main problem is the yeast matrix that is not broken down liberating the vitamin D_2 for intestinal absorption. The present study also used yeast cell wall to try and counteract this matrix effect and overcome the lower bioavailability. However, our results suggest vitamin D_2 from the yeast cell wall fraction did not have much better bioavailability than vitamin D_2 from the intact yeast cells.

We wished to discount the possibility that the lack of increase of serum total 25(OH)D in the young women in the Itkonen et al [8] study who received bread made with UV-treated intact

yeast, was due to enhanced catabolism of 25(OH)D. Thus, we measured serum 24,25(OH)D_2 and its ratio to 25(OH)D in the rats in all nine groups as indices of vitamin D catabolism. 24,25(OH)D_2 is the first catabolite of 25(OH)D arising from its hydroxylation by cytochrome P24A1 (CYP24A1) [20]. In the present study of young growing rats, the 24,25$(OH)_2$D was associated with 25(OH)D concentrations: the more vitamin D ingested, the higher the concentrations of 25(OH)D and the catabolite in serum (in line with that observed in humans [21]). Furthermore, the trend in the differences was similar to that seen in 25(OH)D. The ratio of 24,25$(OH)_2$D to 25(OH)D depends on the activity of CYP24A1, and it has been suggested to be a better marker of vitamin D status than only 25(OH)D, as it seems to be an indicator of vitamin D deficiency [20]. The concentration of 24,25$(OH)_2$D in humans usually ranges from 2 to 20 % of total 25(OH)D [21]. In the present study, however, the proportions in rats were much higher, the values ranging from 69 to 114%, suggesting difference in vitamin D metabolism in rats. Interestingly, the lowest ratios were present in the control group and higher dose vitamin D_3 group, which may indicate that less vitamin D is "wasted" with higher dose of vitamin D_3 whereas no differences were present between different vitamin D_2 sources. However, this does not explain the results of the control group. While acknowledging possible species differences in absolute concentration of 24,25$(OH)_2$D, and its ratio to 25(OH)D, that these were not higher in the yeast-treated groups compared to vitamin D_2 supplement rat groups, might suggest that the observed differences in serum total 25(OH)D between the yeast-derived vitamin D_2 and vitamin D_2 supplement groups in the human trial of Itkonen et al [8] were not due to the increased catabolism, but more likely as a consequence of low bioavailability of the vitamin D_2 from yeast. However, this issue of poor bioavailability of vitamin D_2 from UV-treated foods, seems not to be the case for UV-treated mushrooms. For example, in a rat study with high vitamin D doses in the form of UV-treated mushrooms, Calvo et al [18] found that these mushrooms were effective in increasing plasma

total 25(OH)D. Yet, the study also lacked of a vitamin D_2 or D_3 control. UVB-treated mushrooms, as well the wild mushrooms exposed to UV sunlight, have been shown to be as effective as D_2 supplement in increasing serum total 25(OH)D in humans [22,23].

In our study, bone area and BMC tended to be higher in the vitamin D_3 treated groups than in other groups. This may indicate the positive effect of vitamin D_3 supplementation on bone development, as seen in earlier rat studies [24,25]. However, recently Chun et al [26] found that vitamin D_2 treated mice had higher bone volume/total volume and trabecular number and thickness than vitamin D_3 treated mice. They also found higher free 25(OH)D concentrations in vitamin D_2 fed animals. Free 25(OH)D has been suggested to be a one of the better alternative measures to describe the active, bioavailable part of 25(OH)D and thus vitamin D status [27,28]. Free 25(OH)D has been shown to correlate better than total 25(OH)D with health outcomes such as bone parameters in humans [28]. Unfortunately, in the current study we did not have data on free 25(OH)D. Concerning calcium metabolism markers, while there were no significant differences in S-Ca, unexpectedly both S-Pi and S-PTH were elevated in vitamin D_3-treated groups. Usually 25(OH)D and S-PTH concentrations correlate inversely [29] but in our study the correlation was positive. The extremely high S-PTH concentrations in high dose vitamin D_3 group may be a consequence of the increased S-Pi, interplaying with the active metabolite of vitamin D, serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) [30]. On the other hand, S-PTH concentrations showed notable within group variations, even though analysis was performed in duplicate, and this needs to be borne in mind when interpreting the findings.

The present study used LC-MS/MS technology to measure 25(OH)D_2 and 25(OH)D_3 to generate a serum total 25(OH)D as well as 24,25$(OH)_2D_{2/3}$ as an index of vitamin D catabolism, which was a particular strength. However, the study is not without limitations. Small group sizes might have reduced the statistical significance of the experiment regarding the bone parameters, but these were secondary outcome measures. Furthermore, a longer duration would have provided more information about the long-term exposure to high vitamin D intake and the toxicity. It is also possible that there would have been differences in measured bone parameters if the rats have been allowed to reach the skeletal maturity. Moreover, the blood samples were taken only in the end of the intervention. The baseline information could have offered interesting data on changes in total 25(OH)D and other metabolites. It is also possible that inclusion of vitamin D-deficient rats in the present study, may have led to greater differences in bioavailability between the different vitamin D forms. Further, analysis of free and bioavailable 25(OH)D as well as 1,25$(OH)_2$D could have provided more explanation to the remaining open questions about the bioavailability of the different yeast forms.

In conclusion, the present study in young growing rats did not find major differences between UV-treated vitamin D_2-rich intact yeast cells or the cell wall fraction in terms of their capacity for increasing total serum 25(OH)D, and our findings do not support the hypothesis that the cell wall fraction might be more capable of improving vitamin D status compared to the intact yeast cells. Further, the yeast forms were less potent in increasing serum total 25(OH)D than vitamin D_3 or higher dose vitamin D_2 supplements. These vitamin D status results could not be explained by the increased vitamin D catabolism in the yeast treated groups, where the concentrations of 24,25$(OH)_2$D were similar to or lower than the vitamin D_2 supplement-

448 treated groups. In the light of the present knowledge, UV-treated vitamin D_2-rich yeast could
449 only be recommended as a minor option in terms of a food fortificant until further
450 investigations ascertain the bioavailability of different yeast preparations.
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452
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Figure captions

Figure 1. Biomarkers and bone parameters in the treatment groups: **A** serum 25-hydroxyvitamin D (S-25(OH)D), **B** serum 24,25-dihydroxyvitamin D (S-24,25(OH)D_2), **C** 24,25(OH)D_2 to 25(OH)D ratio, **D** bone mineral content, **E** bone area, **F** bone mineral density, **G** serum phosphate (S-Pi), **H** serum parathyroid hormone (S-PTH), and **I** serum calcium (S-Ca). Values are means ± SE, adjusted for rat body weight. P value in the figure is for the whole test, analysis of covariance. Symbols show significant difference in the *post hoc* comparison (least significant difference, $P<0.05$) between the treatment groups and a: control, b: D_3 supplement 300 IU/d, c: D_3 supplement 600 IU/d, d: D_2 supplement 300 IU/d, e: D_2 supplement 600 IU/d, f: intact yeast cell 300 IU/d, g: intact yeast cell 600 IU/d, h: yeast cell wall 300 IU/d, i: yeast cell wall 600 IU/d. n=6 per treatment group, except n=5 for S-24,25(OH)D_2 and 24,25(OH)D_2 to 25(OH)D ratio.

Table 1 - Ingredient composition of the diets fed to rats (g/kg diet)

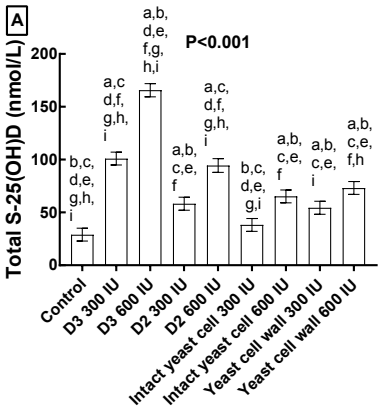
Treatment group and the estimated daily vitamin D dose	Control (D_3 supplement) 25 IU/d	D_3 supplement 300 IU/d	D_3 supplement 600 IU/d	D_2 supplement 300 IU/d	D_2 supplement 600 IU/d	Intact yeast cell 300 IU/d	Intact yeast cell 600 IU/d	Yeast cell wall 300 IU/d	Yeast cell wall 600 IU/d
Form of vitamin D	D_3	D_3	D_3	D_2	D_2	D_2	D_2	D_2	D_2
Amount of vitamin D in the diet (g/kg)	0.000025	0.000375	0.00075	0.000375	0.00075	0.000375	0.00075	0.000375	0.00075
Ingredients (g/kg)									
Casein, "Vitamin-Free" Test	200	200	200	200	200	200	200	200	200
L-Cystine	3	3	3	3	3	3	3	3	3
Corn Starch	397.486	397.206	396.906	397.131	396.756	396.551	395.596	397.236	396.966
Sucrose	99.822	99.822	99.822	99.822	99.822	99.822	99.822	99.822	99.822
Maltodextrin	132	132	132	132	132	132	132	132	132
Cellulose	50	50	50	50	50	50	50	50	50
Soybean Oil	70	70	70	70	70	70	70	70	70
Mineral Mix, AIN-93G-MX (94046)	35	35	35	35	35	35	35	35	35
Vitamin Mix, AIN-93 w/o A, D, E (120379)	10	10	10	10	10	10	10	10	10
Vitamin A Palmitate (500,000 IU/g)	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
TBHQ, antioxidant	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Vitamin D_2, ergocalciferol (40,000 IU/g)	-	-	-	0.375	0.75	-	-	-	-
Vitamin D_2 intact yeast cell (15,700 IU/g)	-	-	-	-	-	0.955	1.91	-	-
Vitamin D_2 yeast cell wall (55,400 IU/g)	-	-	-	-	-	-	-	0.27	0.54
Vitamin D_3, cholecalciferol (50,000 IU/g in sucrose)	0.02	0.3	0.6	-	-	-	-	-	-

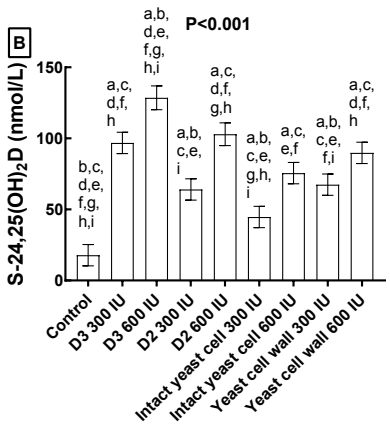
All diets: protein 18.3%, carbohydrate 60.1%, fat 7.0 % by weight. Energy 3.8 Kcal/g.

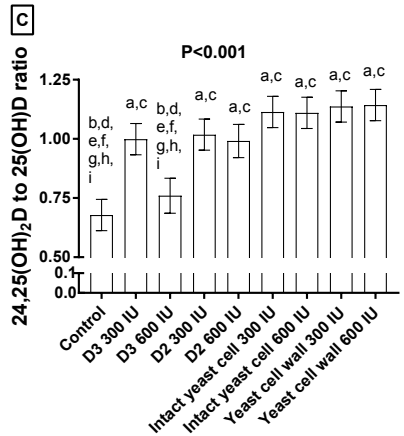
Table 2 - Vitamin D intakes and body weights at endpoint, stratified by the vitamin D treatment group

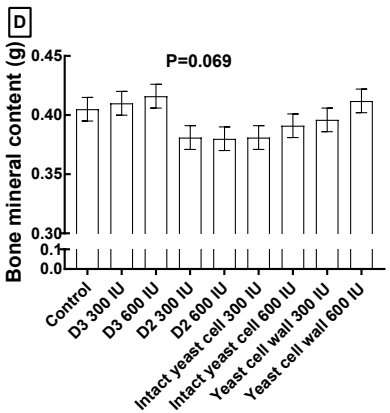
	Control (D_3 supplement) 20 IU/d	D_3 supplement 300 IU/d	D_3 supplement 600 IU/d	D_2 supplement 300 IU/d	D_2 supplement 600 IU/d	Intact yeast cell 300 IU/d	Intact yeast cell 600 IU/d	Yeast cell wall 300 IU/d	Yeast cell wall 600 IU/d	P value[a]
Actual vitamin D intake (IU/d)[b]	14	204	412	205	458	213	412	213	439	na
Actual vitamin D intake (µg/d)[b]	0.35	5.1	10.3	5.1	11.5	5.3	10.3	5.3	11.0	na
Rat body weight (g)[c]	262 ± 5	263 ± 16	253 ± 39	262 ± 29	273 ± 18	273 ± 20	267 ± 25	262 ± 25	270 ± 15	0.317

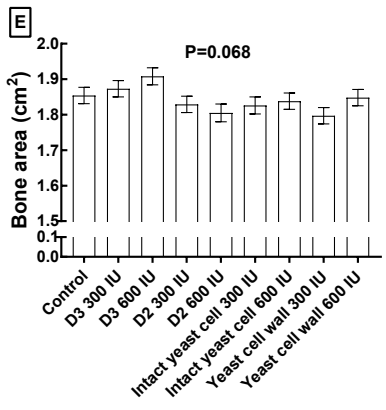
[a] P value from one-way analysis of variance.
[b] Values are group means calculated based on feed consumption, data not available for individual rats.
[c] Values are means ± SD.
na not applicable (the data available per treatment group, not for individual rats).
n = 6 for each group.

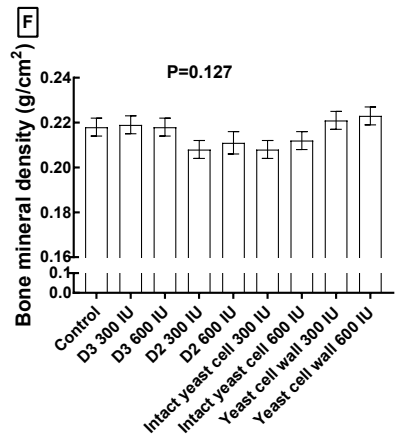

A
Total S-25(OH)D (nmol/L)
P<0.001
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU

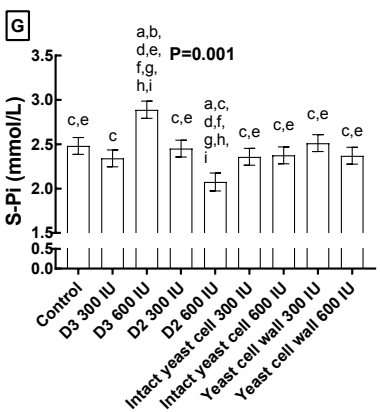

B
S-24,25(OH)2D (nmol/L)
P<0.001
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU

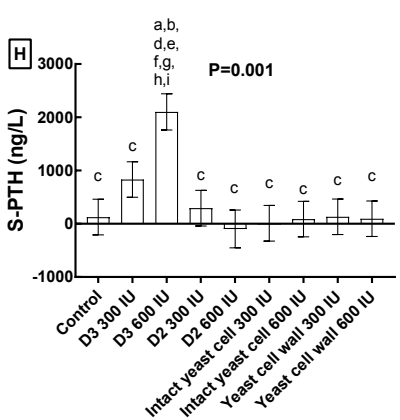

C
24,25(OH)2D to 25(OH)D ratio
P<0.001
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU

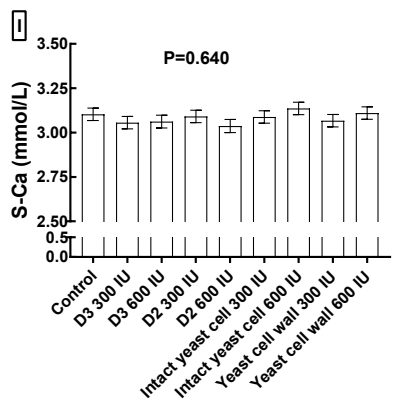

D
Bone mineral content (g)
P=0.069
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU


E
Bone area (cm2)
P=0.068
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU


F
Bone mineral density (g/cm2)
P=0.127
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU


G
S-Pi (mmol/L)
P=0.001
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU


H
S-PTH (ng/L)
P=0.001
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU


I
S-Ca (mmol/L)
P=0.640
Control
D3 300 IU
D3 600 IU
D2 300 IU
D2 600 IU
Intact yeast cell 300 IU
Intact yeast cell 600 IU
Yeast cell wall 300 IU
Yeast cell wall 600 IU