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Oral Intake of Lumisterol Affects the Metabolism of Vitamin D

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Scope: The treatment of food with ultraviolet-B (UV-B) light to increase the vitamin D content is accompanied by the formation of photoisomers, such as lumisterol$_2$. The physiological impact of photoisomers is largely unknown.
Methods and Results: Three groups of C57Bl/6 mice are fed diets containing 50 µg kg^{-1} deuterated vitamin D$_3$ with 0, 50 (moderate-dose) or 2000 µg kg^{-1} (high-dose) lumisterol$_2$ for four weeks. Considerable quantities of lumisterol$_2$ and vitamin D$_2$ are found in the plasma and tissues of mice fed with 2000 µg kg^{-1} lumisterol$_2$ but not in those fed 0 or 50 µg kg^{-1} lumisterol$_2$. Mice fed with 2000 µg kg^{-1} lumisterol$_2$ showed strongly reduced deuterated 25-hydroxyvitamin D$_3$ (–50%) and calcitriol (–80%) levels in plasma, accompanied by downregulated mRNA abundance of *cytochrom P450 (Cyp)27b1* and upregulated *Cyp24a1* in the kidneys. Increased tissue levels of vitamin D$_2$ were also seen in mice in a second study that are kept on a diet with 0.2% UV-B exposed yeast versus those fed 0.2% untreated yeast containing iso-amounts of vitamin D$_2$.
Conclusion: High doses of lumisterol$_2$ can enter the body, induce the formation of vitamin D$_2$, reduce the levels of 25(OH)D$_3$ and calcitriol and strongly impact the expression of genes involved in the degradation and synthesis of bioactive vitamin D.

1. Introduction

Many people worldwide are affected by vitamin D deficiency or insufficiency.[1] Because natural food sources, except oily fish, are usually low in vitamin D, the fortification of food with vitamin D has been considered a viable option to improve vitamin D intake in humans.[2] One strategy to enrich food with vitamin D is the addition of a defined quantity of vitamin D to a food product. An alternative strategy is the irradiation of food with ultraviolet-B light (UV-B).[3] Baker's yeast (*Saccharomyces cerevisiae*) and cultivated mushrooms contain high quantities of ergosterol, the precursor of vitamin D$_2$. Exposure of these foods to UV-B light leads to the conversion of ergosterol to vitamin D$_2$. In 2014, the European Food Safety Authority (EFSA) suggested the use of UV-B treated baker's yeast as a novel ingredient to enrich food, e.g., bakery products with vitamin D$_2$.[4] Another authorized UV-B treated food product are mushrooms.[5] However, the treatment of these foods with UV-B light is accompanied by the formation of photoisomers, such as lumisterol and tachysterol. Among them, lumisterol is quantitatively the most important byproduct of UV-B mediated vitamin D synthesis.[6,7] In their scientific opinions on the safety of UV treated foods, the EFSA noted that the quantities of lumisterol and tachysterol in UV-B exposed foods are low.[4,8,9] Based on these facts, they concluded that photoisomers of vitamin D are not harmful to health or associated with relevant safety concerns.[4,8–10] Wittig et al. demonstrated that mushrooms that were exposed to UV-B light for 60 min contained 78.8 µg of vitamin D$_2$ and 41.1 µg of lumisterol$_2$ per gram of dry matter.[11]

Photoisomers, which can also be formed endogenously from the vitamin D precursor 7-dehydrocholesterol (7-DHC) in the skin by UV-B exposure, are structural vitamin D analogs.[12,13] Vitamin D photoisomers have been shown to exert antiproliferative activity in keratinocytes,[14] but they do not seem to have effects on systemic vitamin D metabolism.[15,16] However, less is known about the effects of oral photoisomer intake. Photoisomers and vitamin D have similar chemical structures and may compete for transporters and enzymes that are involved in tissue uptake, plasma transport and hydroxylation.[12,17] In this context, one relevant intestinal transporter is Niemann-Pick C1-like protein 1 (NPC1L1), which mediates not only the intestinal uptake of cholesterol[18], but also the absorption of vitamin D$_3$.[19,20] Other relevant transporters that appear to be involved in the uptake of vitamin D are cluster of differentiation 36 (CD36) and scavenger receptor class B type I (SRB1, encoded by the Scarb gene).[21] In addition, micelle formation could also be influenced by photoisomers. Previous data have shown that phytosterols that are structurally similar to vitamin D can significantly affect vitamin

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D_3 incorporation into micelles and in turn reduce the apical uptake of vitamin D_3 in Caco-2 cells.[17] It is also conceivable that vitamin D and photoisomers compete for enzymes that catalyze the synthesis of bioactive and inactive vitamin D metabolites. Structural vitamin D analogs are known to be selective inhibitors of hydroxylases in vivo.[22,23] There is evidence from in vivo and in vitro studies that lumisterol$_3$ can function as a substrate for enzymes involved in vitamin D metabolism.[13,24] Slominski et al. showed that lumisterol$_3$, which is hydroxylated by the cytochrome P450 (CYP) 11A1 enzyme, can also serve as a substrate for CYP27B1, CYP27A1 and CYP24A1, enzymes that usually mediate the synthesis of active and inactive vitamin D metabolites.[25,26] While renal CYP27B1 catalyzes the formation of bioactive 1,25-dihydroxyvitamin D (1,25(OH)$_2$D),[27] CYP27A1 and CYP2R1 are important liver enzymes that are involved in the hydroxylation of vitamin D to 25-hydroxyvitamin D (25(OH)D), the primary biomarker of vitamin D status.[28,29] In contrast, CYP24A1 is responsible for the degradation of hydroxylated vitamin D metabolites.[30]

To our knowledge, no study has investigated the intestinal uptake and impact of orally administered vitamin D photoisomers on vitamin D metabolism in a mouse model. Therefore, we conducted two studies: the first study addressed the question of whether oral lumisterol$_2$, the main photoisomer formed in UV-B exposed baker's yeast, can enter the body and affect vitamin D metabolism; the second study was conducted to investigate the impact of UV-B exposed baker's yeast containing a mixture of lumisterol$_2$ and tachysterol$_2$ on vitamin D status in mice.

2. Experimental Section

2.1. Animals and Diets

Mice in both studies were cared for and handled according to the guidelines established by the US National Research Council.[31] The experimental procedures were approved by the committee for animal welfare of the Martin Luther University Halle-Wittenberg (approval number of study 1: H1-4/T4-18; approval number of study 2: H1-4/T2-18). All mice were housed in Macrolon cages in pairs with a 12-h light/12-h dark cycle in a UV-free room at a controlled temperature (22 ± 2 °C) and relative humidity (50–60%).

In both studies, mice received a semi-synthetic basal diet (**Table 1**), supplemented with adequate vitamins and minerals according to the recommendations of the US National Research Council.[32] The diets were kept refrigerated until they were fed to the mice. Food and water were provided ad libitum in both studies.

Study 1 aimed to investigate the uptake of oral lumisterol$_2$ and its effect on orally administered vitamin D. For this purpose, 36 4-week-old male C57Bl/6NCrl mice (Charles River, Sulzfeld, Germany) with an initial body weight of 21.7 ± 1.05 g were randomly allocated to three groups (n = 12) and were fed basal diets supplemented with 50 µg kg^{-1} triple-deuterated vitamin D_3 (vitamin D_3-d_3, Cambridge Isotope Laboratories, Andover, USA) with either 0 (control), 50 (moderate), or 2000 µg kg^{-1} (high) lumisterol$_2$ (Toronto Research Chemicals Inc., North York, ON, Canada) for four weeks. The analyzed concentrations of lumisterol$_2$ in the two lumisterol$_2$ diets were 56 and 1871 µg kg^{-1}. The chosen lumisterol$_2$ concentrations represent moderate levels (5.45 ± 0.24 µg kg^{-1} body weight) and high levels of lumisterol$_2$ (223 ± 6.12 µg kg^{-1} body weight), respectively. As lumisterol$_2$ can probably be reconverted to vitamin D_2 by a thermal reaction, the study additionally analyzed vitamin D_2 in the diet and found 15 µg kg^{-1} vitamin D_2 in the high-lumisterol$_2$ diet and no detectable vitamin D_2 (limit of quantification 0.84 µg kg^{-1}) in the moderate-lumisterol$_2$ and control diets. The analyzed concentrations of lumisterol$_2$ and vitamin D_2 in the diets were examined in triplicate.

Table 1. Composition of the basal diets in study 1 and study 2.

	Study 1 [g kg^{-1}]	Study 2 [g kg^{-1}]
Starch	388	386
Casein	200	200
Sucrose	200	200
Soya oil	100	100
Vitamin-mineral mixture[a)]	60	60
Cellulose	50	50
DL-methionine	2	2
Yeast	0	2
Calcium	5	5
Phosphorus	3	3

[a)] Per kg diet: sodium, 500 mg; chloride, 500 mg, magnesium, 500 mg; iron, 35 mg; manganese, 10 mg, zinc, 10 mg, copper, 6 mg, iodine, 150 µg; molybdenum, 150 µg; selenium, 150 µg; choline, 2 g; tocopherol, 22 mg; vitamin A, 720 µg; vitamin K, 1 mg; pantothenic acid, 16 mg; niacin, 15 mg; vitamin B_6, 8 mg; vitamin B_2, 7 mg; vitamin B_1, 5 mg; folate, 500 µg; biotin, 200 µg, vitamin B_{12}, 10 µg; vitamin D, 50 µg in study 1 (added as triple-deuterated D_3) and 36 µg in study 2 (added as vitamin D_2 from UV-B-exposed baker's yeast or synthetic vitamin D_2)

Study 2 investigated the impact of UV-B-exposed versus unexposed baker's yeast containing the same amounts of vitamin D_2 on the plasma and tissue levels of D-vitamers in mice. The study was conducted with 24 4-week-old male C57BL/6NCrl mice (Charles River, Sulzfeld, Germany) with an initial body weight of 19.8 ± 1.47 g. The mice were randomly allotted into two groups (n = 12) and were fed the basal diets with either 2 g kg^{-1} unexposed baker's yeast (Yeast-UVB) or 2 g kg^{-1} UV-B irradiated baker's yeast (Yeast+UVB) for 5 weeks. The latter was produced by exposure of commercially available baker's yeast to UV-B light with an intensity of 1150 µW cm^{-2} (UV-8M, Herolab GmbH, Wiesloch, Germany) for 30 min. The analyzed concentrations of vitamin D_2, lumisterol$_2$, and tachysterol$_2$ in 2 g of UV-B exposed baker's yeast used for one kg diet were 36, 38, and 1.2 µg, respectively. The control diet, which contained the unexposed baker's yeast, was supplemented with 36 µg kg^{-1} vitamin D_2 to ensure iso-amounts of vitamin D_2 in both diets.

Individual body weights of mice from both studies were recorded weekly. The food intake per cage was recorded daily. After the experimental periods, the mice were deprived of food for four h, anesthetized with diethyl ether and exsanguinated by decapitation. Blood was collected in heparinized or serum tubes (Sarstedt, Nümbrecht, Germany), centrifuged to isolate plasma or serum and stored at −20 °C until analysis. Tissue samples were harvested, immediately snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

Further, the study examined the temperature-dependent conversion of lumisterol$_2$ to vitamin D$_2$. Therefore, standardized quantities of lumisterol$_2$, dissolved in ethanol, were thermally treated at 20 °C and 60 °C, respectively, for two, four, and eight h. Additionally, one aliquot of the lumisterol$_2$ solution was preexposed to UV-B light (intensity of 1150 µW cm^{-2}; UV-8M, Herolab GmbH, Wiesloch, Germany) for 30 min and then analyzed for subsequent changes in the lumisterol$_2$ and vitamin D$_2$ concentrations. Finally, the diet containing the UV-B exposed yeast was thermally treated at 37 °C (to simulate the body temperature) for 24, 48, and 72 h, respectively, to determine vitamin D$_2$ formation.

2.2. Methods

2.2.1. Analysis of D-Vitamers in Diets and Mice

The concentrations of lumisterol$_2$, vitamin D$_2$, vitamin D$_3$, vitamin D$_3$-d$_3$, 25(OH)D$_2$, 25(OH)D$_3$ and triple-deuterated 25-hydroxyvitamin D$_3$ (25(OH)D$_3$-d$_3$) were measured by high-performance liquid chromatography (HPLC; 1260 Infinity Series, Agilent Technologies, Waldbronn, Germany) coupled to an electrospray ionization tandem mass spectrometer (MS/MS, QTRAP 5500, SCIEX, Darmstadt, Germany). To quantify the concentrations of D-vitamers in the diets and tissues of mice treated with vitamin D$_3$-d$_3$ (study 1), sevenfold deuterated vitamin D$_3$ (Toronto Research Chemicals Inc.) and sixfold deuterated 25(OH)D$_3$ (Chemaphor Chemical Services, Ottawa, Canada) were added to the samples as internal standards. To quantify the concentrations of D-vitamers in the diets and tissues of mice treated with baker's yeast and undeuterated vitamin D (study 2), triple-deuterated vitamin D$_3$ (Sigma-Aldrich, Munich, Germany), triple-deuterated vitamin D$_2$ (Sigma-Aldrich), sixfold deuterated 7-DHC (Chemaphor Chemical Services) and sixfold deuterated 25(OH)D$_3$ (Chemaphor Chemical Services) were added to the samples as internal standards.

The mixtures were saponified with potassium hydroxide, extracted with n-hexane and washed with ultrapure water. After evaporation of the solvents, plasma and serum samples were immediately derivatized with 4-phenyl-1,2,4-triazolin-3,5-dione (PTAD, Sigma-Aldrich, Munich, Germany).[33] Diets and tissues were dissolved in n-hexane/isopropanol (99:1 v/v) and purified by normal-phase HPLC (1100 Series, Agilent Technologies) as described elsewhere.[34,35] Two fractions that contained lumisterol$_2$, tachysterol$_2$, vitamin D$_2$, vitamin D$_3$, vitamin D$_3$-d$_3$ or 25(OH)D$_2$, 25(OH)D$_3$, 25(OH)D$_3$-d$_3$ and the corresponding internal standards were collected, dried and derivatized with PTAD.[33] The derivatized samples were dissolved in methanol, mixed with a 10 mmol L^{-1} ammonium formate solution (4:1, v/v, Sigma-Aldrich) and analyzed by LC–MS/MS.

Two different HPLC columns were used for chromatographic separation prior to mass spectrometric analyses. To quantify the photoisomers and vitamin D, the HPLC system was equipped with a Kinetex C18 column (100 Å, 2.6 µm, 100 × 2.1 mm^3, Phenomenex, Torrance, USA); the mobile phase consisted of (A) acetonitrile and (B) a mixture of acetonitrile/water (1:1, v/v) with 5 mmol L^{-1} ammonium formate and 0.1% formic acid. The column temperature and gradient were used as described elsewhere.[19] To quantify 25(OH)D, the HPLC system was equipped with a Hypersil ODS C18 column (120 Å, 5 µm, 150 × 2.0 mm^3, VDS Optilab, Berlin, Germany) and set to a column temperature of 40 °C with a flow rate of 576 µL min^{-1}. The mobile phase consisted of (A) acetonitrile and (B) a mixture of acetonitrile/water as previously described. The following gradient was used: 0.0–3.1 min, 85.0% B; 4.0 min, 83.5% B; 5.0 min, 65.0% B; 7.0–10.0 min, 0% B; 11.0 min, 95.0% B, 12.0–17.0 min, 85.0% B. Ionization for mass spectrometric analyses was induced by positive electrospray ionization, and data were recorded in multiple reaction monitoring (MRM) mode with the following transitions (quantifier ions) [M + PTAD + H$^+$]: lumisterol$_2$, 572 > 395; tachysterol$_2$, 572 > 395; vitamin D$_2$, 572 > 298; vitamin D$_2$-d$_3$, 575 > 301; vitamin D$_3$, 560 > 298; vitamin D$_3$-d$_3$, 563 > 301; sevenfold deuterated vitamin D$_3$, 567 > 279; 25(OH)D$_2$, 588 > 298; 25(OH)D$_3$, 576 > 298; 25(OH)D$_3$-d$_3$, 579 > 301; sixfold deuterated 25(OH)D$_3$, 582 > 298. All mass transitions of the analytes were verified by qualifier ions (lumisterol$_2$, 572 > 377; tachysterol$_2$, 572 > 377; vitamin D$_2$, 572 > 280; vitamin D$_3$, 560 > 280; vitamin D$_3$-d$_3$, 563 > 283; 25(OH)D$_2$, 588 > 341; 25(OH)D$_3$, 576 > 558; 25(OH)D$_3$-d$_3$, 579 > 561). Calibration curves were constructed using external standards of lumisterol$_2$ (Toronto Research Chemicals), vitamin D$_2$ (Sigma-Aldrich), vitamin D$_3$ (Sigma-Aldrich), vitamin D$_3$-d$_3$ (Cambridge Isotope Laboratories), 25(OH)D$_2$ (Enzo Life Sciences Inc., Lörrach, Germany), 25(OH)D$_3$ (Sigma-Aldrich) and 25(OH)D$_3$-d$_3$ (Sigma-Aldrich) spiked with internal standards (as described above). The calibration curve was constructed with a linear regression model (r > 0.99) by plotting the ratio of the analyte peak area to the internal standard peak area versus the concentration of the analytes.

The limit of quantification (LOQ) for plasma and serum samples was 1.2 nmol L^{-1} for lumisterol$_2$, 0.5 nmol L^{-1} for vitamin D$_2$, 0.3 nmol L^{-1} for vitamin D$_3$ and 6.1 nmol L^{-1} for 25(OH)D$_2$ and 25(OH)D$_3$. The LOQ for tissue samples was 7.3 ng g^{-1} for lumisterol$_2$, 1.0 ng g^{-1} for vitamin D$_2$, 0.5 ng g^{-1} for vitamin D$_3$, 2.2 ng g^{-1} for 25(OH)D$_2$, 3.8 ng g^{-1} for 25(OH)D$_3$, and 2.6 ng g^{-1} for 25(OH)D$_3$-d$_3$. The LOQ for diet samples was 3 µg kg^{-1} for lumisterol$_2$ and 0.84 µg kg^{-1} for vitamin D$_2$. Precision of the method was assessed by repeat measures of pooled diet samples (n = 10) and liver samples (n = 8). Precision data are shown in **Table 2**.

2.2.2. Analysis of Circulating 1,25(OH)$_2$D, Parathyroid Hormone and Cholesterol in Mice

The plasma concentration of 1,25(OH)$_2$D was analyzed by a commercial enzyme-linked immunoassay (Immunodiagnostic Systems, Frankfurt am Main, Germany), and parathyroid hormone (PTH) was measured by a two-site enzyme-linked immunosorbent assay (Immunotopics, San Clemente, USA). A commercial photometric assay was used to quantify plasma concentrations of cholesterol (Cholesterol FS, Diagnostic Systems GmbH, Holzheim, Germany). All analyses were performed by following the procedures given by the manufacturers with modifications.[36]

2.2.3. Analysis of the Relative mRNA Abundance of Genes Involved in the Uptake and Metabolism of Vitamin D

The relative mRNA abundance of vitamin D target genes and genes involved in vitamin D and sterol metabolism in the

Table 2. Precision of vitamin D analysis.

	Coefficient of variation (%)		
	Intra-day precision (repeat measures)		Precision of instruments[c]
	Diet[a]	Tissue[b]	
Lumisterol$_2$	36.5*	18.8*	14.3*
Vitamin D$_2$	7.41	4.95	2.84
Vitamin D$_3$-d$_3$	8.61	6.69	6.56
25(OH)D$_3$-d$_3$	n.r.	11.0	5.50

n.r., not relevant; vitamin D$_3$-d$_3$, triple-deuterated vitamin D$_3$; 25(OH)D$_3$-d$_3$, triple-deuterated 25-hydroxyvitamin D$_3$. The intra-day precision was determined by repeated measurements of each vitamin D metabolite in the diet (n = 10) and liver (n = 8). The precision of instruments was determined by ten consecutive injections of a representative sample. [a] Metabolite concentrations used to assess the intra-day precision: lumisterol$_2$, 76.7 ng g^{-1}; vitamin D$_2$, 12.3 ng g^{-1}; vitamin D$_3$-d$_3$, 36.9 ng g^{-1}; [b] Metabolite concentrations used to assess the intra-day precision: lumisterol$_2$, 149 ng g^{-1}; vitamin D$_2$, 91.3 ng g^{-1}; vitamin D$_3$-d$_3$, 47.2 ng g^{-1}; 25(OH)D$_3$-d$_3$, 28.0 ng g^{-1}; [c] Metabolite concentrations used to assess the precision of instruments: lumisterol$_2$, 144 ng g^{-1}; vitamin D$_2$, 98.5 ng g^{-1}; vitamin D$_3$-d$_3$, 46.3 ng g^{-1}; 25(OH)D$_3$-d$_3$, 42.8 ng g^{-1}. *The high coefficient of variation was partly caused by the instability of lumisterol$_2$ during sample preparation and analysis.

Table 3. Primer sequences used for the analyses of the relative mRNA abundance of genes.

Gene	Obtained from	Annealing [°C]	Accession number
Abcg5	Eurofins MWG Synthesis	60	NM_031884.1
Abcg8	Eurofins MWG Synthesis	60	NM_026180.2
B2m[a]	Eurofins MWG Synthesis	60	NM_009735.3
Calb3	Eurofins MWG Synthesis	62	NM_009789.2
Cd36	Eurofins MWG Synthesis	60	NM_001159556.1
Cldn2	Sigma-Aldrich	57	NM_016675
Cyp24a1	Sigma-Aldrich	58	NM_009996.3
Cyp27a1	Sigma-Aldrich	60	NM_024264.4
Cyp27b1	Sigma-Aldrich	58	NM_010009.2
Cyp2j3	Sigma-Aldrich	58	NM_175766.3
Cyp2r1	Sigma-Aldrich	60	NM_177382.4
Cyp3a11	Sigma-Aldrich	60	NM_007818.3
Hprt[a]	Sigma-Aldrich	57	NM_013556.2
Npc1l1	Eurofins MWG Synthesis	60	NM_207242.2
Rplp0[a]	Eurofins MWG Synthesis	60	NM_007475.5
Scarb1	Eurofins MWG Synthesis	60	NM_016741
Trpv6	Sigma Aldrich	64	NM_022413
Vdr	Eurofins MWG Synthesis	60	NM_009504.4

Eurofins MWG Synthesis GmbH, Ebersberg, Germany. Sigma Aldrich, Taufkirchen, Germany. *Abcg5*, ATP-binding cassette subfamily G member 5; *Abcg8*, ATP-binding cassette subfamily G member 8; *B2m*, beta-2 microglobulin; *Calb3, calbindin-D$_{9k}$; Cd36*, cluster of differentiation 36; *Cldn2*, claudin-2; *Cyp24a1*, vitamin D 24-hydroxylase; *Cyp27a1*, sterol 27-hydroxylase; *Cyp27b1*, 25-hydroxyvitamin D-1 α hydroxylase; *Cyp2r1*, vitamin D 25-hydroxylase; *Cyp2j3*, cytochrome P450, family 2, subfamily J, polypeptide 3; *Cyp3a11*, cytochrome P450, family 3, subfamily A, polypeptide 11; *Hprt*, hypoxanthine guanine phosphoribosyl transferase; *Npc1l1*, Niemann-Pick C1-like protein 1; *Rplp0*, ribosomal protein, large, P0; *Scarb1*, scavenger receptor class B type 1; *Trpv6*, transient receptor potential cation channel, subfamily V, member 6; *Vdr*, vitamin D receptor. [a] Reference gene.

tissues of mice was analyzed by real-time RT-PCR. Total RNA was isolated from the liver, kidney and intestinal mucosa with the peqGOLD TriFast Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer´s protocol. The RNA concentration was calculated using a NanoDrop Spectrophotometer (Thermo Fisher Scientific GmbH, Schwerte, Germany), and RNA purity was confirmed by agarose gel electrophoresis. cDNA was synthesized using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). A total of 1 µL of cDNA template was amplified as described elsewhere[37] using GoTaq Flexi DNA-Polymerase (Promega) and the Rotorgene 6000 system (Corbett Research, Mortlake, Australia). According to the RT-PCR protocol, initial denaturation was performed at 95 °C for 3 min, followed by 20–30 cycles of denaturation at 95 °C, annealing at primer-specific temperatures (**Table 3**) and elongation at 72 °C. The amplification of the single and specific mRNAs was confirmed by agarose gel electrophoresis. The calculation of relative mRNA concentration was based on the method of Pfaffl.[38] Beta-2-microglobulin (*B2m*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and the ribosomal protein, large, P0 (*Rplp0*) were used as appropriate reference genes. The relative mRNA abundance of target genes was expressed as the fold change in relation to the control group. The target and reference genes are shown in Table 3.

2.3. Statistical Analysis

Data are expressed as the mean ± standard derivation (SD). Statistical analyses were performed using SPSS version 25.0 (IBM, Armonk, USA). Data from study 1 including three groups of mice were subjected to the Shapiro-Wilk normality and homoscedasticity of variance test (Levene's test). For normally distributed parameters, the groups were compared by one-way analysis of variance (ANOVA). When ANOVA revealed significant differences, data with equally distributed variances were compared with the Tukey-HSD post hoc test. The Games-Howell test was used for data with unequally distributed variances. In the case of non-normally distributed parameters, data were subjected to the nonparametric Kruskal-Wallis test. If the Kruskal-Wallis test revealed significant differences, groups were compared by the Mann-Whitney *U* test for post hoc comparisons. A Bonferroni correction for multiple testing was used. If the metabolite concentration in a sample within a group was below the LOQ, a randomly generated value (between 0 and the appropriate LOQ) was used for statistical treatment analysis. In cases in which all mice within a group had metabolite levels below the LOQ, the data were not included in the statistical analyses. If two of the three groups had metabolite levels above the LOQ, the means of the two groups were analyzed by Student´s *t*-test or the nonparametric Mann-Whitney *U* test. The data were considered significantly different at $p < 0.05$.

The results of study 2 were subjected to a normality test using the Shapiro–Wilk test. If the data followed a normal distribution, differences between the groups were analyzed by Student´s *t*-test. If not, the nonparametric Mann-Whitney *U* test was used. Differences were considered to be significant at $p < 0.05$.

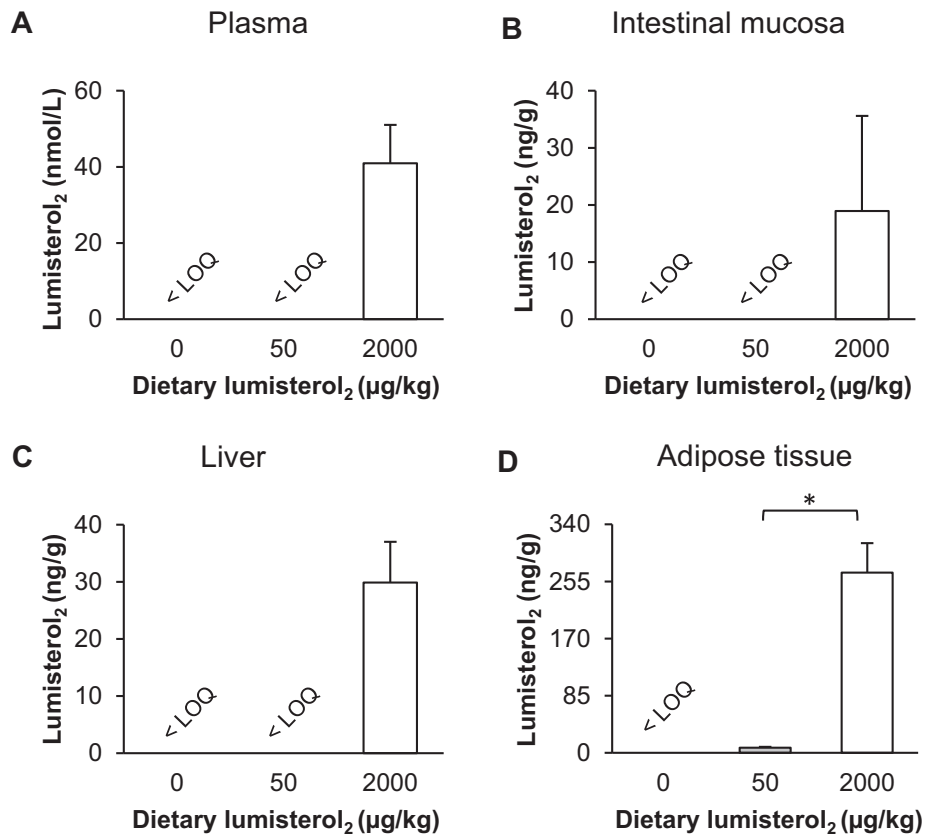

Figure 1. Concentrations of lumisterol$_2$ in the plasma (A), intestinal mucosa (B), liver (C) and retroperitoneal adipose tissue (D) of mice receiving diets with 0, 50 or 2000 µg kg^{-1} lumisterol$_2$ for four weeks. All diets contained 50 µg kg^{-1} triple-deuterated vitamin D$_3$. The data are presented as the mean ± standard deviation (n = 12). Quantifiable data from two groups were compared with Student's *t*-test. * Indicates statistically significant differences ($p < 0.05$). The limit of quantification (LOQ) of lumisterol$_2$ was 1.2 nmol L^{-1} for plasma and 7.3 ng g^{-1} for tissue samples.

3. Results

3.1. Study 1: Impact of Oral Lumisterol$_2$ on Vitamin D Metabolism

3.1.1. Oral Lumisterol$_2$ Did Not Influence the Body Weight or Food Intake of Mice

The final body weights (groups: 0 µg kg^{-1}, 27.5 ± 1.11 g; 50 µg kg^{-1}, 26.2 ± 2.12 g; 2000 µg kg^{-1}, 27.0 ± 2.26 g) and mean daily food intake assessed by two mice per cage (groups: 0 µg kg^{-1}, 2.93 ± 0.47 g; 50 µg kg^{-1}, 2.85 ± 0.41 g; 2000 µg kg^{-1}, 3.00 ± 0.40 g) did not differ between the three groups of mice treated with 0, 50 or 2000 µg lumisterol$_2$ per kg diet.

3.1.2. Oral Lumisterol$_2$ Can Enter the Body

To determine whether oral lumisterol$_2$ can enter the body, we analyzed the levels of lumisterol$_2$ in the plasma, intestinal mucosa, liver, retroperitoneal adipose tissue and kidney of mice. Detectable concentrations of lumisterol$_2$ were observed in plasma and all tissues, except kidney, of mice that received 2000 µg kg^{-1} lumisterol$_2$ (**Figure 1**A–D). In mice fed 50 µg kg^{-1} lumisterol$_2$, detectable levels of lumisterol$_2$ were found only in the retroperitoneal adipose tissue (Figure 1D), whereas the lumisterol$_2$ concentrations in the plasma, intestinal mucosa, liver and kidney were below the LOQ (Figure 1A–C). Mice receiving the control diet had no detectable lumisterol$_2$ in plasma or any tissues analyzed.

3.1.3. Orally Administered Lumisterol$_2$ was Partly Converted to Vitamin D$_2$ in Mice

As photoisomers can be reconverted to vitamin D$_2$ via thermoconversion, we analyzed the concentrations of vitamin D$_2$ and hydroxylated D$_2$-vitamers in the plasma and tissues of mice fed lumisterol$_2$. Considerable quantities of vitamin D$_2$ in plasma and all tissues analyzed were observed in mice fed 2000 µg kg^{-1} lumisterol$_2$ (**Figure 2**A–E). In mice fed 50 µg kg^{-1} lumisterol$_2$, low levels of vitamin D$_2$ were found in the retroperitoneal adipose tissue, whereas in the plasma, intestinal mucosa, liver and kidney, the vitamin D$_2$ concentrations were below the LOQ. Mice receiving the control diet had no detectable vitamin D$_2$ in plasma or any tissues analyzed.

To determine whether vitamin D$_2$ formed in the high-lumisterol$_2$ group was hydroxylated to 25(OH)D$_2$, we analyzed the concentrations of 25(OH)D$_2$ in the plasma and tissues of mice. Detectable concentrations of 25(OH)D$_2$ were found in the plasma and kidneys of mice that received 2000 µg kg^{-1}

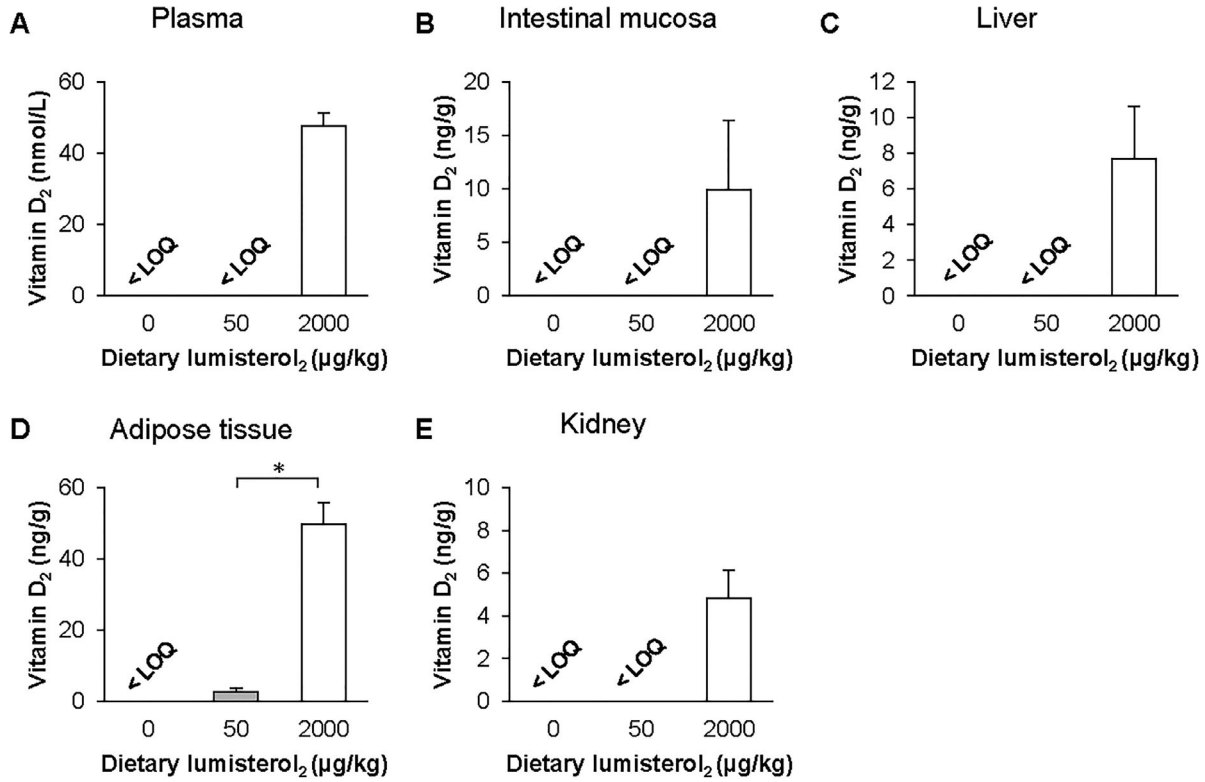

Figure 2. Concentrations of vitamin D_2 in the plasma (A), intestinal mucosa (B), liver (C), retroperitoneal adipose tissue (D) and kidney (E) of mice receiving diets with 0, 50 or 2000 µg kg^{-1} lumisterol$_2$ for four weeks. All diets contained 50 µg kg^{-1} triple-deuterated vitamin D_3. The data are presented as the mean ± standard deviation (n = 12). Quantifiable data from two groups were compared with Student's *t*-test. * Indicates statistically significant differences ($p < 0.05$). The limit of quantification (LOQ) of vitamin D_2 was 0.5 nmol L^{-1} for plasma and 1.0 ng g^{-1} for tissue samples.

lumisterol$_2$ but not in the groups fed 0 or 50 µg kg^{-1} lumisterol$_2$ (**Figure 3**A,B).

3.1.4. Oral Lumisterol$_2$ Slightly Increases Vitamin D_3-d_3 levels in Tissues

To investigate the impact of oral lumisterol$_2$ on the uptake and tissue levels of labeled vitamin D_3, we determined the levels of vitamin D_3-d_3 in the plasma and tissues of mice. The concentrations of vitamin D_3-d_3 in the plasma of both groups of mice that received lumisterol$_2$ were comparable to those in the controls, although the concentration of vitamin D_3-d_3 was higher in the plasma of mice fed diets with 2000 µg kg^{-1} lumisterol$_2$ than in mice fed 50 µg kg^{-1} lumisterol$_2$ (**Figure 4**A). Interestingly, we found that the vitamin D_3-d_3 levels were higher in the intestinal mucosa of mice fed diets with 50 and 2000 µg kg^{-1} lumisterol$_2$

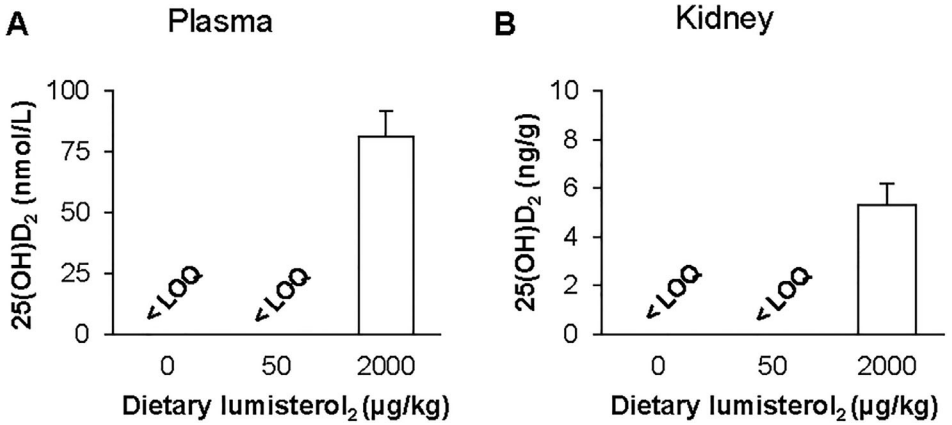

Figure 3. 25(OH)D_2 concentrations in the plasma (A) and kidney (B) of mice receiving diets with 0, 50 or 2000 µg kg^{-1} lumisterol$_2$ for four weeks. All diets contained 50 µg kg^{-1} triple-deuterated vitamin D_3. The data are presented as the mean ± standard deviation (n = 12). The limit of quantification (LOQ) of 25(OH)D_2 was 6.1 nmol L^{-1} for plasma and 2.2 ng g^{-1} for tissue samples. 25(OH)D_2, 25-hydroxyvitamin D_2.

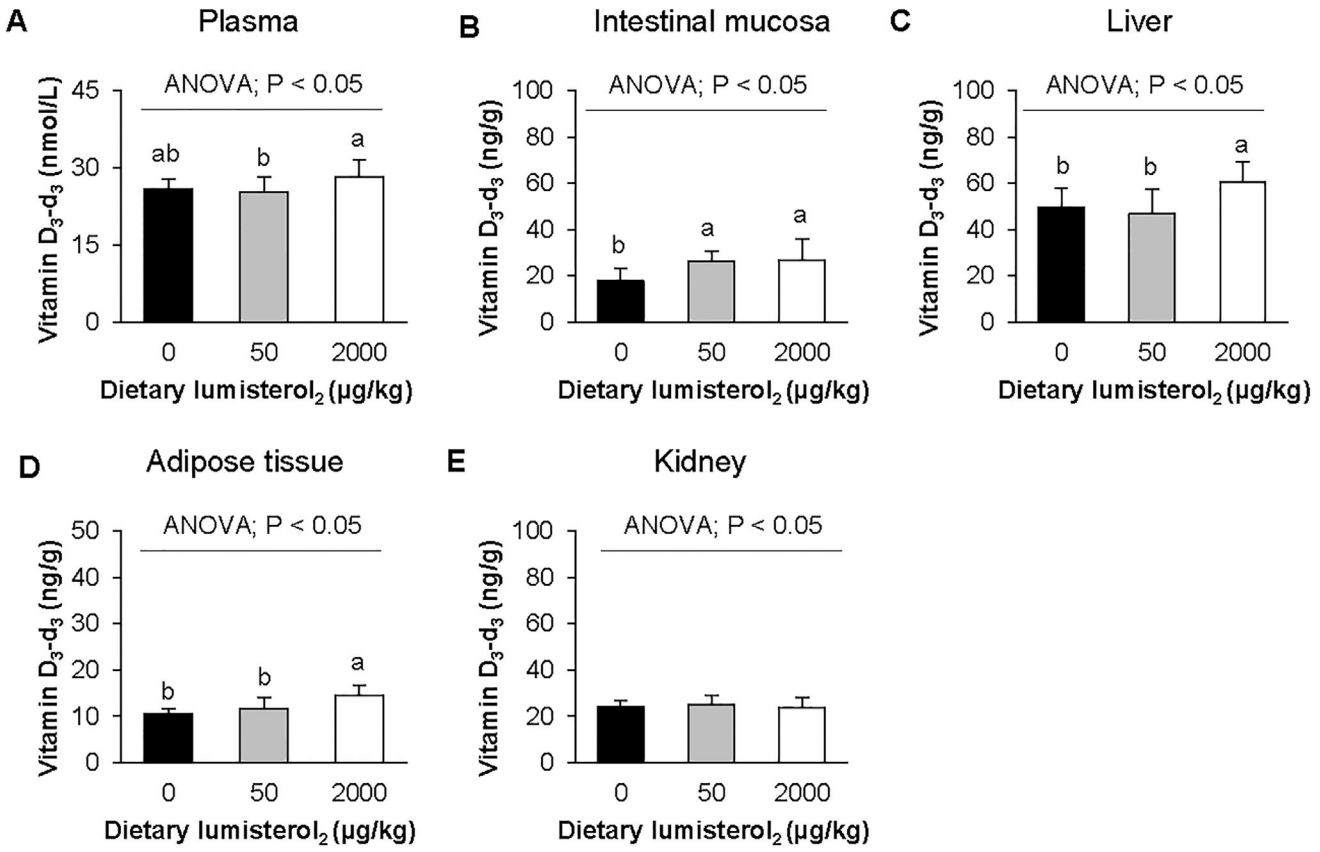

Figure 4. Vitamin D_3-d_3 concentrations in the plasma (A), intestinal mucosa (B), liver (C), retroperitoneal adipose tissue (D) and kidney (E) of mice receiving diets with 0, 50 or 2000 µg kg^{-1} lumisterol$_2$ for four weeks. All diets contained 50 µg kg^{-1} triple-deuterated vitamin D_3. The data are presented as the mean ± standard deviation (n = 12) and were analyzed by one-way analysis of variance (ANOVA). Different letters indicate statistically significant differences ($p < 0.05$) among the three groups of mice. Vitamin D_3-d_3, triple-deuterated vitamin D_3.

than in the controls (Figure 4B). In the liver and retroperitoneal adipose tissue, increased levels of vitamin D_3-d_3 were only found in the group fed 2000 µg kg^{-1} lumisterol$_2$, whereas no differences were observed between the group fed 50 µg kg^{-1} lumisterol$_2$ and the controls (Figure 4C,E). The levels of vitamin D_3-d_3 in the kidney remained unaffected by the treatments (Figure 4D).

Finally, we ascertained the levels of non-deuterated vitamin D_3 and found vitamin D_3 only at low concentrations in the liver (groups: 0 µg kg^{-1}, 1.27 ± 0.83 ng g^{-1}; 50 µg kg^{-1}, 1.47 ± 0.93 ng g^{-1}; 2000 µg kg^{-1}, 1.52 ± 1.03 ng g^{-1}). The vitamin D_3 concentrations in the plasma, intestinal mucosa, kidney and retroperitoneal adipose tissue were below the LOQ.

To examine the impact of dietary lumisterol$_2$ on vitamin D transporters, we analyzed ATP binding cassette transporters G5 and G8 (*Abcg5/g8*) and the *Cd36*, *Npc1l1* and *Scarb1* mRNA abundance in the intestinal mucosa. The data indicate that the *Npc1l1* mRNA abundance was increased in the group fed 2000 µg kg^{-1} lumisterol$_2$ compared to the other two groups (**Table 4**). Differences in the mRNA abundance of *Abcg5* were only seen between mice fed 2000 and 50 µg kg^{-1} lumisterol$_2$ but not between both lumisterol$_2$ groups and the control (Table 4). No differences in the mRNA abundance of *Abcg8*, *Cd36* and *Scarb1* were found among the three groups of mice (Table 4).

3.1.5. Oral Lumisterol$_2$ Affects the Formation of 25(OH)D_3-d_3

The data demonstrate that the concentration of 25(OH)D_3-d_3 was strongly reduced in the plasma, liver and kidney in mice fed 2000 µg kg^{-1} lumisterol$_2$ compared to mice fed 0 and 50 µg kg^{-1} lumisterol$_2$ (**Figure 5**A–C). The concentration of 25(OH)D_3-d_3 did not differ between the groups fed 0 and 50 µg kg^{-1} lumisterol$_2$.

To provide possible causes for the alterations in the levels of 25(OH)D_3-d_3, we analyzed the mRNA abundance of the most important hepatic genes that are involved in the hydroxylation of vitamin D but found no differences in the mRNA abundance of *Cyp27a1, Cyp2r1, Cyp2j3*, and *Cyp3a11* in the livers of mice from the three groups (Table 4).

3.1.6. Oral Lumisterol$_2$ Affects the Synthesis of the Bioactive Vitamin D Hormone

Next, we quantified circulating levels of 1,25$(OH)_2$D in plasma and found that the group fed 2000 µg kg^{-1} lumisterol$_2$ had considerably lower concentrations of 1,25$(OH)_2$D than the other two groups (**Figure 6**A). No differences in 1,25$(OH)_2$D levels were observed between the groups fed 0 and 50 µg kg^{-1} lumisterol$_2$. To

Table 4. Relative mRNA abundance of hepatic genes involved in the hydroxylation of vitamin D or intestinal vitamin D transport, and vitamin D receptor target genes of mice fed diets with different quantities of lumisterol$_2$.

	Lumisterol$_2$ in µg kg^{-1} diet			
	0	50	2000	*p*
Liver				
Cyp27a1	1.00 ± 0.14	0.94 ± 0.22	0.86 ± 0.18	n.s.
Cyp2r1	1.00 ± 0.23	1.12 ± 0.23	0.96 ± 0.24	n.s.
Cyp3a11	1.00 ± 0.17	1.00 ± 0.33	0.81 ± 0.17	n.s.
Cyp2j3	1.00 ± 0.21	1.28 ± 0.36	1.21 ± 0.39	n.s.
Intestinal mucosa				
Abcg5	1.00 ± 0.28^{ab}	0.95 ± 0.27^{b}	1.25 ± 0.34^{a}	<0.05
Abcg8	1.00 ± 0.21	0.97 ± 0.24	1.18 ± 0.31	n.s.
Calb3	1.00 ± 0.85	0.80 ± 0.37	0.78 ± 0.38	n.s.
Cd36	1.00 ± 0.29	1.06 ± 0.35	1.02 ± 0.24	n.s.
Cldn2	1.00 ± 0.59	0.98 ± 0.48	0.83 ± 0.59	n.s.
Npc1l1	1.00 ± 0.19^{a}	1.01 ± 0.19^{a}	1.33 ± 0.31^{b}	<0.05
Scarb1	1.00 ± 0.68	0.99 ± 0.99	1.08 ± 0.58	n.s.
Trpv6	1.00 ± 0.87	0.90 ± 0.46	1.31 ± 1.34	n.s.
Vdr	1.00± 0.18	1.10 ± 0.25	1.12 ± 0.26	n.s.

All diets contained 50 µg kg^{-1} triple-deuterated vitamin D$_3$. The data are presented as the mean ± standard deviation (n = 12) and were analyzed by one-way analysis of variance (ANOVA). Different letters indicate statistically significant differences ($p < 0.05$) between the three groups of mice. n.s.: not significant. *Cyp27a1*, sterol 27-hydroxylase; *Cyp2r1*, vitamin D 25-hydroxylase; *Cyp2j3*, cytochrome P450, family 2, subfamily J, polypeptide 3; *Cyp3a11*, cytochrome P450, family 3, subfamily A, polypeptide 11; *Abcg5*, ATP-binding cassette subfamily G member 5; *Abcg8*, ATP-binding cassette subfamily G member 8; *Calb3*, calbindin-D$_{9k}$; *Cd36*, cluster of differentiation 36; *Cldn2*, claudin-2; *Npc1l1*, Niemann-Pick C1-like protein 1; *Scarb1*, scavenger receptor class B type 1; *Trpv6*, transient receptor potential cation channel, subfamily V, member 6; *Vdr*, vitamin D receptor

determine whether the reduced level of 1,25(OH)$_2$D in mice fed 2000 µg kg^{-1} lumisterol$_2$ represents a vitamin D-deficient state, we analyzed the plasma concentrations of PTH in the mice but did not find any differences among the three groups of mice (Figure 6B).

To ascertain whether the reduced plasma level of bioactive vitamin D hormone was associated with impaired vitamin D action, we analyzed the mRNA abundance of the vitamin D receptor (*Vdr*) and classic vitamin D receptor target genes, including calbindin D$_{9k}$ (*S100g*), claudin-2 (*Cldn2*), and transient receptor potential cation channel, subfamily V, member 6 (*Trpv6*), in the intestinal mucosa but did not find differences among the three groups of mice (Table 4).

Gene expression data demonstrated a marked reduction in *Cyp27b1* mRNA abundance and a strong increase in *Cyp24a1* mRNA abundance in the group fed 2000 µg kg^{-1} lumisterol$_2$ compared to the other two groups (Figure 6C,D).

3.2. Study 2: Impact of UV-B-Exposed Baker's Yeast on D-Vitamers in Mice

3.2.1. Oral Intake of UV-B-Exposed Yeast did not Influence the Body Weight or Food Intake of Mice

The final body weights (Yeast-UVB, 24.9 ± 1.51 g; Yeast+UVB, 25.2 ± 2.27 g) and mean daily food intake assessed for two mice per cage (Yeast-UVB, 2.64 ± 0.31 g; Yeast+UVB, 2.69 ± 0.43 g) did not differ between the two groups of mice.

3.2.2. UV-B-Exposed Yeast Increased the Levels of Vitamin D$_2$ in the Intestinal Mucosa and Liver

This study compared the impact of diets containing either UV-B-exposed yeast or unexposed yeast containing iso-amounts of vitamin D$_2$ on D-vitamer levels in mice. First, we measured the concentrations of the photoisomers lumisterol$_2$ and tachysterol$_2$ in the serum and tissues of mice but found no detectable quantities of either photoisomer in the serum, intestinal mucosa, liver or kidney in either group. The serum concentrations of vitamin D$_2$, 25(OH)D$_2$ and 1,25(OH)$_2$D did not differ between the two groups (**Figure 7**A–C). However, we found approximately 1.3-times higher vitamin D$_2$ concentrations in the intestinal mucosa

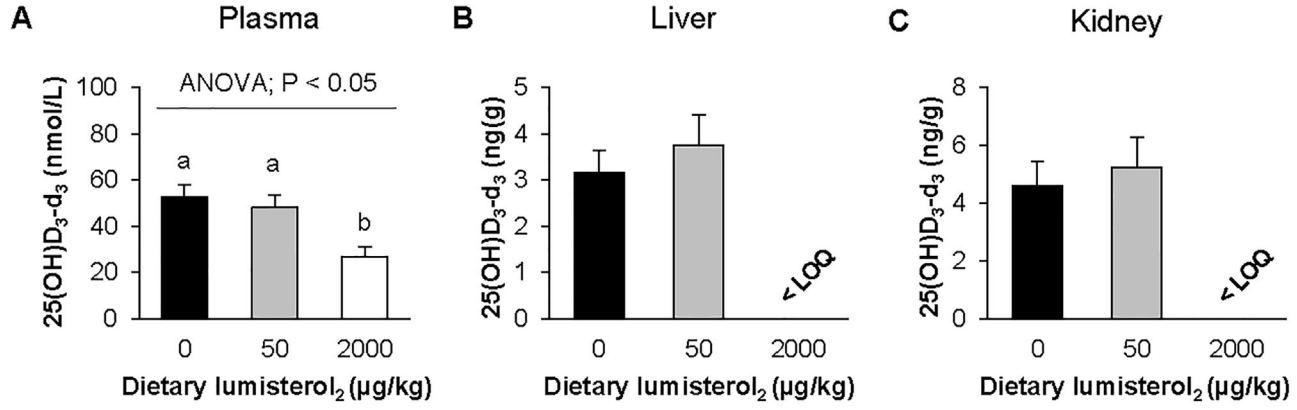

Figure 5. 25(OH)D$_3$-d$_3$ concentrations in the plasma (A), liver (B) and kidney (C) of mice receiving diets with 0, 50 or 2000 µg kg^{-1} lumisterol$_2$ for four weeks. All diets contained 50 µg kg^{-1} triple-deuterated vitamin D$_3$. The data are presented as the mean ± standard deviation (n = 12). Data from three groups were analyzed by one-way analysis of variance (ANOVA). Quantifiable data from two groups were compared with Student's *t*-test. Different letters indicate statistically significant differences ($p < 0.05$) among the three groups of mice. The limit of quantification (LOQ) of 25(OH)D$_3$-d$_3$ was 2.6 ng g^{-1}. 25(OH)D$_3$-d$_3$, triple-deuterated 25-hydroxyvitamin D$_3$.

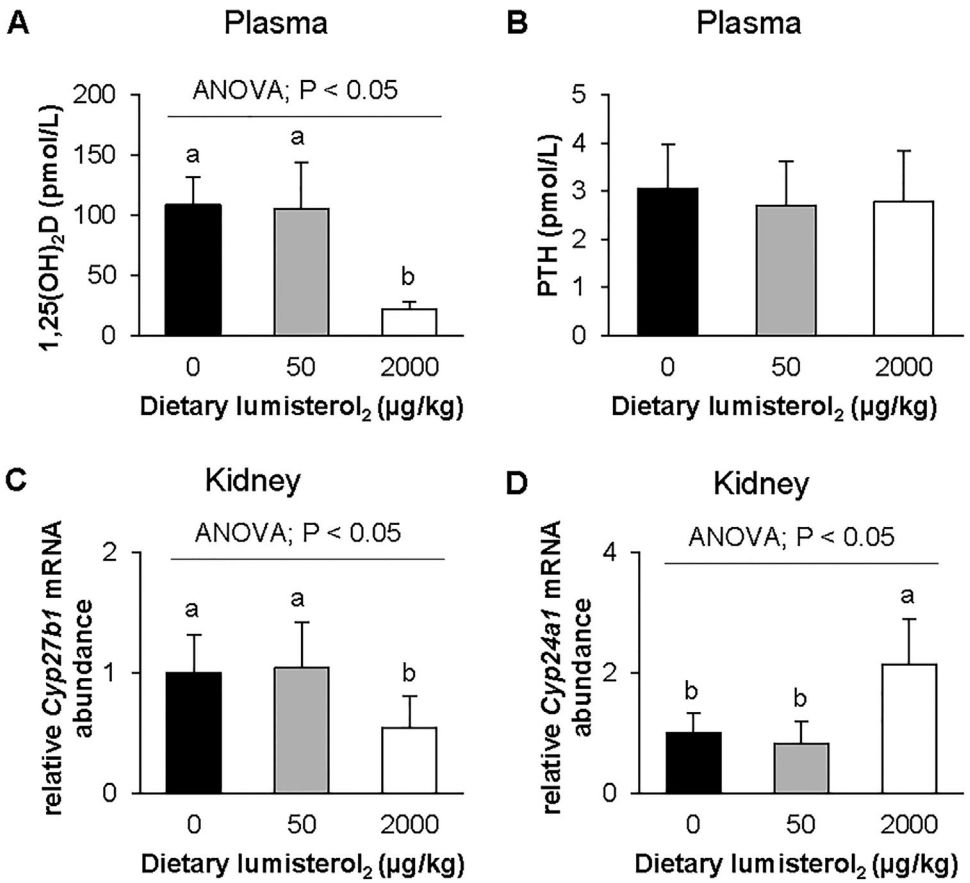

Figure 6. Plasma concentrations of $1,25(OH)_2D$ (A) and PTH (B) and relative mRNA abundance of *Cyp27b1* (C) and *Cyp24a1* (D) in the kidneys of mice receiving diets with 0, 50 or 2000 µg kg^{-1} $lumisterol_2$ for four weeks. All diets contained 50 µg kg^{-1} triple-deuterated vitamin D_3. The data are presented as the mean ± standard deviation (n = 12) and were analyzed by one-way analysis of variance (ANOVA). Different letters indicate statistically significant differences ($p < 0.05$) among the three groups of mice. $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; *Cyp27b1*, 25-hydroxyvitamin D-1 α hydroxylase; *Cyp24a1*, vitamin D 24-hydroxylase; PTH, parathyroid hormone.

and liver of mice fed UV-B exposed yeast than in those of mice that received unexposed yeast (Figure 7D,E). The concentration of vitamin D_2 in the kidney did not differ between the two groups (Figure 7F).

3.3. Temperature- and UV-B Light-Induced Conversion of $Lumisterol_2$ to Vitamin D_2

To investigate the temperature-dependent conversion of $lumisterol_2$ to vitamin D_2, ethanolic solutions of $lumisterol_2$ were subjected to 20 °C and 60 °C for eight h, respectively. Additionally, to determine whether the pretreatment of $lumisterol_2$ with UV-B light can also stimulate the subsequent vitamin D_2 formation, $lumisterol_2$ was exposed to UV-B light for 30 min. All treatments resulted in a reduction of $lumisterol_2$ (**Figure 8**A) and an increase of vitamin D_2 (Figure 8B). The strongest increase of vitamin D_2 was found when $lumisterol_2$ was pretreated with UV-B light, followed by the 60 °C and 20 °C treatments (Figure 8B). Interestingly, the formation of vitamin D_2 did not reach a plateau within 8 h after the $lumisterol_2$ pretreatment with UV-B light.

To determine whether the higher concentrations of vitamin D_2 in the gut and liver of mice fed UV-B exposed yeast were caused by subsequent conversion of photoisomers to vitamin D_2 in the bodies of the mice, we analyzed the formation of vitamin D_2 at body temperature (37 °C). Here, we found that the treatment of the UV-B-exposed yeast diet at 37 °C for 24, 48, and 72 h increased the concentrations of vitamin D_2 by 8%, 43% and 63%, respectively.

4. Discussion

Exposure of food to UV-B light is a novel approach to enrich food with vitamin D. However, this strategy is accompanied by the formation of photoisomers, such as lumisterol, the most abundant photoisomer in UV-B treated baker's yeast and mushrooms.[6,7,11] To elucidate the impact of this photoisomer on the fate of orally administered vitamin D and vitamin D metabolism, we first conducted a study with mice that received 50 µg kg^{-1} deuterium-labeled vitamin D_3 with 0, 50 or 2000 µg kg^{-1} $lumisterol_2$. Interestingly, we found that high doses of $lumisterol_2$ can enter the body and exert pronounced effects on vitamin D metabolism.

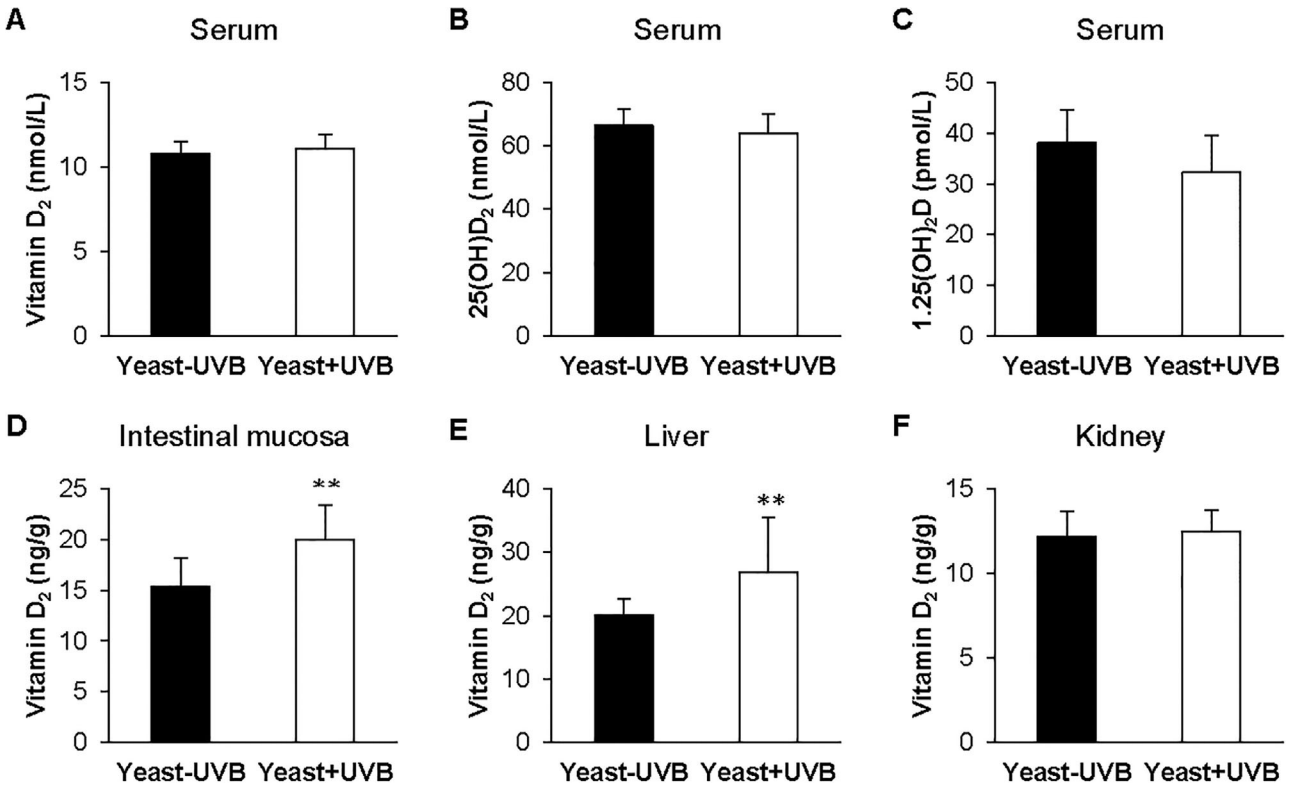

Figure 7. Serum concentrations of vitamin D_2 (A), 25(OH)D_2 (B) and 1,25(OH)$_2$D (C) in mice. Vitamin D_2 concentrations in the intestinal mucosa (D), liver (E) and kidney (F) of mice receiving diets containing UV-B exposed yeast (Yeast+UVB) or unexposed yeast containing iso-amounts of vitamin D_2 (Yeast-UVB) for five weeks. The data are presented as the mean ± standard deviation (n = 12) and were compared by Student's *t*-test. **Indicates statistically significant differences ($p < 0.01$).

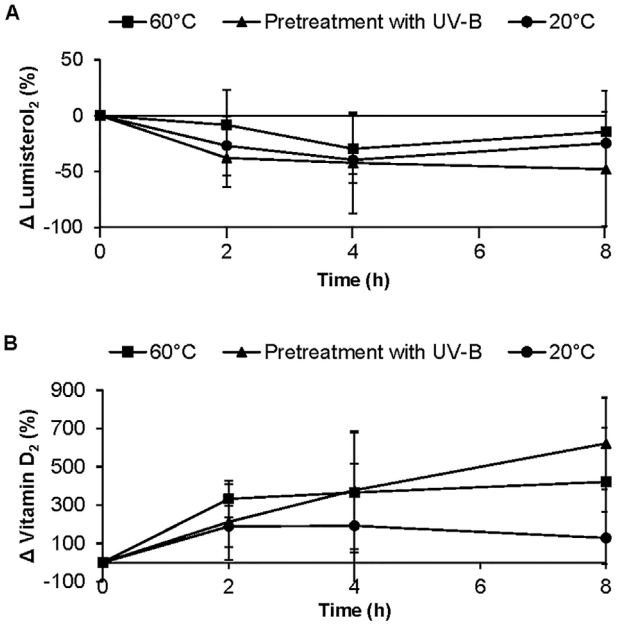

Figure 8. Time-dependent changes (in %) of lumisterol$_2$ (A) and vitamin D_2 (B) during the thermal treatment of lumisterol$_2$ at 20 °C and 60 °C, respectively, and after pretreatment of lumisterol$_2$ with UV-B light for 30 min (20 °C). The treatment experiments were conducted immediately after mixing cooled lumisterol$_2$ with ethanol at room temperature. The data are presented as the mean ± standard deviation (n = 3).

Most noticeably, there was a strong increase in vitamin D_2 content in the plasma and tissues of mice fed the high-lumisterol$_2$ diet. Vitamin D_2 can be formed from lumisterol$_2$ by a thermal reaction.[39,40] Analysis of the diet enriched with 2000 µg kg^{-1} lumisterol$_2$ revealed vitamin D_2 concentrations of 15 µg kg^{-1}, indicating that small amounts of lumisterol$_2$ were converted to vitamin D_2 during the preparation of the diet. This may explain the increase in vitamin D_2 levels in mice fed the high-lumisterol$_2$ diet. While the vitamin D_2 concentrations in the intestinal mucosa, liver and kidney were lower than the corresponding levels of vitamin D_3-d_3 in these tissues, the vitamin D_2 concentrations in the adipose tissue and plasma were markedly higher than those of vitamin D_3. More importantly, mice fed the diet with 2000 µg kg^{-1} lumisterol$_2$ had higher plasma concentrations of 25(OH)D_2 than of 25(OH)D_3-d_3. We therefore hypothesize that a subsequent temperature-dependent isomerization of lumisterol$_2$ to vitamin D_2 took place in the bodies of these mice, which caused an increase in vitamin D_2 levels in plasma and tissues. The detection of vitamin D_2 in the adipose tissue of the group fed the low-lumisterol$_2$ diet corroborates this hypothesis because no detectable vitamin D_2 was found in the diet containing 50 µg kg^{-1} lumisterol$_2$. In addition, the conversion studies with lumisterol$_2$ indicate a distinct temperature-dependent formation of vitamin D_2 from lumisterol$_2$.

To determine whether photoisomers formed in UV-B-exposed baker's yeast may also contribute to a rise in vitamin D_2 levels in mice, we compared in study 2 the serum and tissue levels of

D_2 in mice that were fed identical vitamin D_2 amounts via UV-B exposed yeast and vitamin D_2-enriched unexposed yeast. The increased concentrations of vitamin D_2 in the intestinal mucosa and liver observed in the mice fed the UV-B exposed yeast are indicative of such stimulated vitamin D_2 formation in the bodies of these mice. The experimental data which showed a pronounced conversion of lumisterol$_2$ to vitamin D_2, particularly in the case of prior UV-B irradiation, support the assumption that lumisterol$_2$ can be converted to vitamin D_2 several hours after the UV-B exposure. Additionally, within a day, we observed an increase in dietary vitamin D_2 content when the environmental temperature was switched from 22 °C to 37 °C.

In addition to vitamin D_2, mice from study 1 that were fed the high-lumisterol$_2$ diet had higher levels of vitamin D_3-d_3 in tissues but lower levels of 25(OH)D_3-d_3 and the bioactive vitamin D hormone 1,25(OH)$_2$D than mice fed no lumisterol$_2$. The observed increase in tissue levels of vitamin D_3-d_3 was surprising because we hypothesized that lumisterol$_2$ and vitamin D_3 may compete for intestinal uptake due to their similar chemical structures. It is important to note that recent data from our group also showed an increase in tissue levels of vitamin D_3 when feeding mice 7-DHC[35] or ergosterol,[36] which have structures closely related to that of vitamin D. To determine whether intestinal uptake may explain the increased tissue levels of vitamin D_3-d_3 in mice fed the high dose of lumisterol$_2$, we analyzed the transcription levels of potential vitamin D transporters. These included the cholesterol transporters NPC1L1[19,20]; CD36, which facilitates fatty acid uptake[41,42] and chylomicron formation[43]; SRB1[20]; and the reverse sterol transporters ABCG5/G8, which mediate the export of xenosterols, such as phytosterols.[44] Recently, we demonstrated a strong decrease in the tissue levels of vitamin D_3-d_3 after treatment of mice with the NPC1L1 inhibitor ezetimibe, suggesting that NPC1L1 is the most important vitamin D transporter in the gut.[19] As we found a higher mRNA abundance of NPC1L1 in the intestinal mucosa of mice treated with the high lumisterol$_2$ dose, we hypothesize that lumisterol$_2$ or the converted vitamin D_2 improved the intestinal availability of vitamin D by influencing NPC1L1 at the transcriptional level.

Alternatively, the increased tissue levels of vitamin D_3-d_3 in the high-lumisterol$_2$ group could also result from impaired conversion of vitamin D_3-d_3 to 25(OH)D_3-d_3 by lumisterol$_2$ or vitamin D_2, the latter being found to induce a disproportionately strong reduction in 25(OH)D_3 in mice.[45–48] The liver is the main site for the 25-hydroxylation of vitamin D because it includes a variety of enzymes, including CYP27A1, CYP2R1, CYP3A11 and CYP2J3, which are involved in 25(OH)D synthesis.[46] However, the current data do not indicate any changes in the transcription levels of these enzymes following lumisterol$_2$ treatment. Thus, we hypothesize that liver hydroxylation may not explain the higher vitamin D_3-d_3 and the lower 25(OH)D_3-d_3 levels observed in these mice. The increased mRNA expression of *Cyp24a1*, which we observed in the mice treated with 2000 µg kg^{-1} lumisterol$_2$, was likely responsible for the reduction in 25(OH)D_3-d_3. Consistent with this finding is the concurrent reduction in 1,25(OH)$_2$D because CYP24A1 catalyzes not only the degradation of 25(OH)D but also that of 1,25(OH)$_2$D.[30] The observed impact of oral lumisterol$_2$ on CYP24A1 probably explains the results from human and rat studies that failed to show an increase in 25(OH)D plasma levels after the consumption of bread containing UV-B exposed vitamin D-rich yeast compared to vitamin D supplements.[47,48] The current finding that the circulating level of 25(OH)D_2 in mice fed UV-B exposed yeast did not differ from that of unexposed yeast, although mice fed UV-B exposed yeast had higher levels of vitamin D_2 in the intestinal mucosa and liver, fits well with this hypothesis.

Since we also found a reduced expression of *Cyp27b1*, the key enzyme in the synthesis of 1,25(OH)$_2$D from 25(OH)D,[27] we suggest that the low plasma levels of the bioactive vitamin D hormone 1,25(OH)$_2$D in mice fed the high lumisterol$_2$ dose resulted from increased degradation and reduced synthesis. The bioactive vitamin D hormone 1,25(OH)$_2$D normally binds to the vitamin D receptor, which then forms a dimer with the retinoic acid receptor and stimulates the transcription of proteins, for example, those that are involved in the uptake of calcium.[49] A lack of 1,25(OH)$_2$D usually leads to the synthesis and secretion of PTH.[50,51] Despite having low plasma levels of 1,25(OH)$_2$D, mice fed the high lumisterol$_2$ dose did not show increased PTH levels. Three possible explanations can be considered for this finding. First, it is possible that the reduction in 25(OH)D and 1,25(OH)$_2$D was not severe enough to induce secondary parathyroidism. This hypothesis is supported by recent data from Mallya and coworkers, who found no changes in PTH levels despite inducing low levels of 25(OH)D and 1,25(OH)$_2$D in mice by feeding with vitamin D-deficient diets.[52] Second, it is likely that the hydroxylated lumisterol$_2$ metabolites exerted an active vitamin D effect. This assumption is corroborated by the finding that hydroxylated lumisterol$_3$ metabolites that are formed in keratinocytes have been shown to be able to stimulate the vitamin D receptor.[12] Third, the additionally formed D_2-vitamers likely induced moderate hypervitaminosis D in the mice, which in turn activated the degradation of hydroxylated D-vitamers via CYP24A1 or reduced renal formation of 1,25(OH)$_2$D via CYP27B1.

To ascertain whether the marked decline in circulating 1,25(OH)$_2$D in mice fed the high lumisterol$_2$ dose was accompanied by impaired vitamin D action, we analyzed the mRNA abundance of four classic vitamin D receptor target genes but found no differences among the groups. Thus, the data do not indicate that mice treated with the high lumisterol$_2$ dose had developed vitamin D deficiency.

To estimate the relevance of these studies for humans, the following model calculation was conducted. The presently used doses of lumisterol$_2$ (50 and 2000 µg kg^{-1} diet) equate to 5.45 and 223 µg lumisterol$_2$ per kg body weight of mice. Using the dose translation formula of Reagan-Shaw et al.[53] which is based on the body surface area normalization method, these animal doses correspond to 0.5 and 18 µg lumisterol$_2$ per kg human body weight or the intake of 35 and 1.26 mg lumisterol$_2$, respectively, in a 70 kg person. The question can therefore be posed whether it is possible to achieve such a high intake of lumisterol$_2$ via the consumption of UV-B treated foods. Data on the quantities of photoisomers in UV-B exposed food for human nutrition are scarce, and photoisomer concentrations can be modified by changing the time of treatment and the radiant intensity used for the production of UV-B treated food sources.[54,55] For example, powder of UV-B-treated *Agaricus bisporus* mushrooms whose safety has been assessed by the EFSA contained 206 µg g^{-1} lumisterol$_2$ and 111 µg g^{-1} tachsterol$_2$.[9] Wittig et al.,

who exposed oyster mushrooms (*Pleurotus ostreatus*) to UV-B light, found lumisterol$_2$ at a concentration of 41.1 µg g^{-1} dry matter after an irradiation period of 60 min.[11] Provided, that an adult consumes daily 100 g of these UV-B exposed oyster mushrooms (dry matter content of 10%), the estimated lumisterol$_2$ dose is 0.4 mg kg^{-1} body weight. This corresponds to a value which lies between the two dosages we used in the current study. However, ascending dose studies are necessary to investigate safety and tolerability of multiple doses of lumisterol$_2$ and to specify the cut-off level. Because UV-B exposure of foods becomes increasingly important for improving vitamin D supply, it is important to keep attention to possible side effects of photoisomers, not at least due to the potential of photoisomers to be converted to vitamin D$_2$.

In conclusion, high lumisterol$_2$ doses can modulate vitamin D metabolism. The modulations include an increase in the tissue levels of not only vitamin D$_2$ but also orally administered vitamin D$_3$ and a strong decline in 25(OH)D and the bioactive compound calcitriol. However, these distinct alterations in vitamin D metabolism are seen only when feeding high levels of lumisterol$_2$. Studies 1 and 2 further showed that moderate levels of photoisomers in the food or diet have only a minor impact on vitamin D metabolism. Thus, in the future, the health impact of high photoisomer amounts must be considered when using the UV-B irradiation approach to fortify food with vitamin D.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.K., J.K., A.C.B., and G.I.S. designed the mouse experiments. J.K. performed the mouse experiments. J.K. analyzed the data. J.K. conducted the statistical analysis. J.K. and G.I.S. wrote the manuscript. J.K. and A.C.B. critically reviewed the manuscript.

Data Availability Statement

Data available on request from the authors.

Keywords

25-hydroxyvitamin D, calcitriol, lumisterol$_2$, mice, UV-B irradiated yeast, vitamin D

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