DOI 10.1002/mnfr.201300286 *Mol. Nutr. Food Res.* 2014, *58*, 318–328

RESEARCH ARTICLE

Vitamin D_2 from UVB light exposed mushrooms modulates immune response to LPS in rats

Uma S. Babu , Kannan V. Balan , Larry H. Garthoff and Mona S. Calvo

Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD, USA

Received: April 19, 2013
Revised: June 18, 2013
Accepted: June 19, 2013

Scope: Poor vitamin D (vitD) status is linked to increased risk of infectious diseases, thus there is need for vitD-rich foods. UVB-exposed mushrooms synthesize $vitD_2$ but knowledge of bioavailability and function in immune response is lacking.

Methods and results: One hundred rats were fed one of five diets—control, 20 IU $vitD_3$/day; no $vitD_3$/day; 5% unexposed mushroom, 2.4 IU $vitD_2$/day; 2.5% UVB mushroom, 300 IU $vitD_2$/day; and 5% UVB mushroom, 600 IU $vitD_2$/day—for 10 wk and challenged with either saline or the endotoxin LPS. Blood and tissues were collected at 3 h postchallenge. Plasma 25-hydroxyvitamin D (25OHD) levels from UVB-exposed mushroom fed rats were significantly elevated and associated with higher natural killer cell activity and reduced plasma inflammatory response to LPS compared to control diet fed rats. Microarray evaluation of rat spleens for changes in inflammatory gene expression showed significant upregulation of proinflammatory genes after LPS compared to saline controls in all groups. However, compared to control rats, upregulation of the proinflammatory genes was markedly reduced in the groups fed $vitD_2$-enriched mushrooms.

Conclusion: Rats fed UVB-exposed mushrooms had significantly higher plasma total 25OHD levels that were associated with increased innate immune response and anti-inflammatory effects.

Keywords:
Inflammation / Lipopolysaccharide / Mushrooms / NK activity / Vitamin D2

1 Introduction

The important association between poor vitamin D (vitD) status and increased risk of infectious diseases such as tuberculosis was first made in the early part of the 20th century with the observed health benefits experienced by tuberculosis patients recovering in sunny locations [1, 2]. Conventional thought is that the majority of circulating 25-hydroxyvitamin D (25OHD), the main indicator of vitD nutritional status, originates from cutaneous synthesis of $vitD_3$ upon exposure to adequate sunlight. However, seasonal changes, living at high latitudes or low altitudes with high levels of air pollution, dark skin pigmentation, and aging are among the many factors that can impede this process, thus requiring reliance on dietary sources to supply the precursor to 25OHD [3]. The continuous nationally representative health surveys in the United States (NHANES) and Canada show a high prevalence of poor vitD status as measured by serum 25OHD and low dietary intake of vitD-rich foods [4–6], which has raised concern about the possible adverse effects vitD inadequacy may have on immune response as well as skeletal health [2]. While adverse skeletal effects of poor vitD status are well established [7], we are only beginning to understand its impact on the immune system [1, 2] and the need for more vitD-rich food sources. In most of North America, low vitD intake is largely due to the limited number of foods that are naturally rich in vitD or are fortified with vitD [8, 9]. VitD was listed as one of four critical nutrients that Americans need to consume more as per the Federal Dietary Guidelines for Americans, 2010 (2010 DGA). Recommendations include that efforts to

Correspondence: Dr. Mona S. Calvo, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 8301 Muirkirk Road, Laurel, MD 20708, USA
E-mail: mona.calvo@fda.hhs.gov
Fax: +1-301-210-4669

Abbreviations: Con A, concanavalin A; **CT**, cycle threshold; **MNCs**, mononuclear cells; **NK**, natural killer; **vitD**, vitamin D; **25OHD**, 25-hydroxyvitamin D

meet nutrient needs for vitD as well as other nutrients should be made through food rather than dietary supplements [10].

Two forms of vitD occur in foods, cholecalciferol or D_3 found in some animal products and fortified foods, and ergocalciferol or D_2 from yeast and fungi, such as white button mushrooms when they have been exposed to UVB light [11–14]. Both forms of the vitamin are used in food fortification with some restrictions, such as specific foods consumed by vegetarians and vegans in the US that can only be fortified with $vitD_2$ [15]. For example, vitD fortification of soymilk and other plant- or grain-based milks is restricted to using $vitD_2$ because it is not of animal origin [15]. In developing new foods providing either $vitD_2$ or D_3, both forms of vitD must be absorbed and bioavailable for use in different tissues including immune cells. Bioavailability requires ingested $vitD_2$ or D_3 to be transported after absorption to the liver via chylomicrons where it is hydroxylated and released to the circulation as either $25OHD_2$ or $25OHD_3$, which circulate bound to vitD-binding protein [16]. The intermediate form (25OHD) circulates and delivers the precursor to tissues to be further metabolized to the active hormonal form of vitD (1,25-dihydroxyvitamin D). For these reasons, circulating levels of the intermediate metabolite ($25OHD_2$ or $25OHD_3$) are used both as a nutritional status indicator and a measure of vitD bioavailability [17].

Our study was designed to test the bioavailability of $vitD_2$ from UVB light exposed edible mushrooms that are the only natural food source of $vitD_2$ commonly consumed in the US and Canada [15, 18]. The ability of commonly consumed white button mushrooms (*Agaricus bisporus*) to make ample levels of $vitD_2$ is well established, as it is for other varieties of edible fungi [11–14, 19–21]. We hypothesized that $vitD_2$ from fresh mushrooms exposed to UVB light postharvest would be absorbed, bioavailable, safe, and effective in modulating the inflammatory response to a sublethal challenge with the endotoxin, LPS. Even at intake levels exceeding the dietary recommendations for the rat, the mushroom source of $vitD_2$ supported optimal bone growth without safety concerns such as soft tissue calcification, which we reported in an earlier publication [22]. The use of EDTA-plasma collecting tubes optimized the total blood volume collected in this study, but prevented analyses of plasma total calcium in all the rat samples. We used renal function measures (plasma creatinine concentrations) and histologic staining for soft tissue calcification to confirm safety and the absence of hypercalcemia-induced soft tissue mineralization and impairment of renal function, all manifestations of hypercalcemia from vitD toxicity or elevations in parathyroid hormone (PTH). Here, we report our findings from a 10-wk feeding study that was designed to examine the effects of $vitD_2$ from light-exposed white button mushrooms on the immune response in growing female rats terminally challenged with a sublethal dose of endotoxin. In order to assure a wide margin of safety for human consumption, we tested mushrooms contributing $vitD_2$ over a large dose range exceeding the recommended intake of vitD for rats by 30-fold.

2 Materials and methods

2.1 Diet and experimental design

One hundred weanling female Sprague-Dawley rats (3 wk old, Harlan, Indianapolis, IN) were allowed to acclimate for 1 wk prior to randomization into the study. Rats were fed ad libitum with one of five diets, all formulated based on AIN-93G (Research Diets, New Brunswick, NJ) to contain different levels of $vitD_3$ and D_2 from either ultraviolet light exposed mushroom powder or unexposed mushroom powder as described previously [22]. Powdered, freeze-dried, UVB light exposed, and unexposed white button mushrooms were generously provided by the Australian Mushroom Growers Association through Mushrooms Canada and the Guelph Food Technology Centre, and samples from each contained 15 and <0.5 μg $vitD_2$ per grams dried mushroom, respectively, as analyzed by Dr. Pirjo Mattila, MTT Agrifood, Finland [23]. To more closely resemble the typical high-fat American diet, all diets differed from the standard AIN-93G formula by their 15% fat content in contrast to the standard 5% fat content. The diets contained 15% (150 g/kg diet) American Fat Blend (Research Diets) consisting of 37.5 g cocoa butter, 4.5 g linseed oil, 52.5 g palm oil, 28.5 g safflower oil, and 27.0 g sunflower oil. Control and vitD-deficient diets had no mushroom powder and contained either the recommended level of $vitD_3$ [24] or no $vitD_3$, while the remaining test diets were all made with vitD-free premix and contained 5.0% unexposed mushroom powder, or 2.5 or 5.0% UVB light exposed mushroom powder added at the expense of cornstarch. Estimated vitD content of the five diets was calculated from direct analyses of the mushroom powder. The rats were estimated to consume 20 (control D_3), 0 ($vitD_3$-deficient), 2.4 (unexposed mushroom D_2), 300 (2.5% UVB-exposed mushroom D_2), and 600 IU of vitD/day (5% UVB-exposed mushroom D_2) from each of the experimental diets. The feeding period was 10 wk, at the end of which rat pairs were challenged individually with either intraperitoneal saline or *Escherichia coli* LPS (100 μg/Kg body weight) [25] and necropsied after 3 h. Immediately after death, blood was drawn into K_3EDTA vacutainer tubes, centrifuged for plasma separation, and stored in aliquots at −80°C until analyzed. Spleens were removed immediately and prepared for assessment of immune response as described in Sections 2.2–2.9. The animal protocol was approved by the IACUC committee of the Center for Food Safety and Applied Nutrition of the US Food and Drug Administration, and the study was conducted according to the Animal Welfare Act guidelines.

2.2 Biomarkers of vitD status

The Dia Sorin (Stillwater, MN) radioimmunoassay recognizes both $25OHD_2$ and $25OHD_3$ equally and the outcome measure is rat plasma total 25OHD ($25OHD_2 + 25OHD_3$) concentration in nanograms per milliliter. Interassay variation based on low (9.3–19.7 ng/mL) and high

(50.7–68.9 ng/mL) controls were 15 and 13%, respectively. Rat plasma PTH levels, which are often elevated in vitD deficiency in humans and rats, were determined using ALPCO Diagnostics rat PTH IRMA (Salem, NH). Assay performance based on high controls (336–560 pg/mL) was 10.6 and 15.2% for inter- and intraassay variation, respectively.

2.3 Isolation of mononuclear cells (MNCs) from spleen

Spleen MNCs were isolated as described elsewhere [26]. Briefly, spleens were minced in Hanks' Balanced Salt Solution (Sigma, St. Louis, MO), filtered and the single cell suspension was overlayered on Histopaque (Sigma, density 1.083 g/mL). MNCs were obtained from the interface after centrifugation at about 400 × *g* for 30 min at room temperature. Cells were washed and the final pellet was adjusted to 4×10^6 cells/mL for proliferation assay or to 1×10^7 cells/mL for natural killer (NK) activity measurement and cell subset analyses by flow cytometry.

2.4 NK cell activity

NK activity was measured as described previously [27]. YAC-1 target cells were incubated with approximately 300 μCi [^{51}Cr] $NaCrO_4$ overnight. After washing thoroughly with PBS, the target cells were resuspended in RPMI containing 10% fetal bovine serum at 10^5 cells/mL (Sigma). Target cells (100 μL) were incubated with equal volume of splenic MNCs (effector cells) using the ratio of one target: 25–100 effector cells for approximately 6 h at 37°C in 5% CO_2 atmosphere. Maximum release of radioactivity was measured by adding 100 μL 0.1% Triton-X-100 (Sigma) to target cells. To measure spontaneous release, cells were incubated with RPMI medium (Sigma) without effector cells. Radioactivity released into the supernatant was measured using a microbeta counter. Percent NK cell activity was calculated using the standard formula, [(experimental release−spontaneous release)/(maximum release−spontaneous release)] × 100.

2.5 Mitogenic reactivity in vitro

Mitogenic reactivity to concanavalin A (Con A) and *E. coli* LPS was assessed as described previously with minor modifications [26]. Aliquots of splenic MNCs (2×10^5 cells) in RPMI 1640 medium with 10% fetal bovine serum were incubated with Con A (0.4, 0.8, or 1.6 μg/well) or LPS (2.5, 5.0, or 10 μg/well) for 72 h at 37°C and in a 5% CO_2 atmosphere. At the end of 48 h, cells were incubated with 0.5 μCi ^{3}H-thymidine for an additional 24 h. Cells were harvested using a 96-well automatic harvester and counted in a microbeta counter. Data are calculated as stimulation indices (stimulated count/nonstimulated count). Each stimulation index value was an average of four separate cultures from the same spleen since these assays are inherently prone to variability.

2.6 Immunophenotyping by flow cytometry

Immunophenotyping was carried out using freshly isolated spleen MNCs as described previously [26], using a FACSAria II flow cytometer (BD Biosciences, CA). Briefly, splenic MNCs were incubated with the monoclonal antibodies specific for rat cell surface antigens including $CD3^+$ (total T), $CD4^+$ (T helper/inducer), $CD8^+$ (T suppressor/cytotoxic), $CD45^+$ (regulator of T- and B-cell activation) and $CD25^+$ (T- and B-cell activation marker, IL-2 receptor), for at least 45 min at 4°C. Standard antibody pairing was done for two color analysis. Samples were then washed and fixed until further analysis. Before sample analysis, the flow cytometer was optimized using isotypic IgG controls and 5000 cells were analyzed for each antibody combination.

2.7 Measurement of IL-2 in Con A induced spleen cell supernatants

Splenic MNCs were cultured in the presence or absence of Con A and the supernatants were collected 48 h later and stored at −80°C until analyzed for IL-2 using commercially available kits from R&D Systems (Minneapolis, MN, USA). IL-2 was selected because it is a representative cytokine expressed in helper T cells, which plays an important role in initiating and potentiating a variety of immune responses. IL-2 is produced by activated T lymphocytes and it regulates other immune cells including B cells, macrophages, and NK cells [28]. IL-2-stimulated response was calculated as the difference between the IL-2 detected in Con A stimulated culture and that in the unstimulated cultures (RPMI). The estimated increase in IL-2 is presented as picograms per milliliters supernatant.

2.8 Evaluation of splenic inflammatory responses

The spleen, a hematopoietic organ, plays an important role in innate and adaptive immune responses producing pro- and anti-inflammatory mediators, which are secreted into the general circulation during LPS-induced inflammation, thus making it a representative tissue to measure inflammatory changes in response to various stimuli [29]. To evaluate vitD-mediated anti-inflammatory responses, splenic inflammatory transcriptional responses were evaluated in LPS-treated and saline control rats. At 3 h post intraperitoneal LPS challenge, ten animals per group were anesthetized, exsanguinated via cardiac puncture, and spleens were immediately harvested, washed in sterile PBS and frozen in RNAlater (Ambion, Austin, TX, USA) at −20°C.

2.8.1 RNA isolation and microarray

Total RNA was isolated from the spleen by using an EZ1 RNA Tissue Kit combined with the EZ1 Advanced Automated Instrument (QIAGEN, Valencia, CA). Residual genomic DNA contamination was removed by incorporating RNAse-free DNase in the extraction procedure (QIAGEN). Four micrograms of RNA from each sample was then reverse-transcribed to cDNA using a RT2 First-strand Synthesis Kit (QIAGEN). The quality of the cDNA conversion was confirmed by running a Quality Control PCR array (QIAGEN), to confirm the absence of genomic DNA, and contaminants that will inhibit reverse transcription and PCR amplification. To quantitate splenic inflammatory transcriptional responses, the expression levels of 84 cytokines and chemokine-like molecules were evaluated using the RT2 Profiler Rat Inflammatory Cytokines PCR Array System (QIAGEN) and CFX96 Real-time PCR Detection System (Life Science Research, Hercules, CA, USA). In order to normalize the data, five endogenous control genes namely, ribosomal protein, large, P1, hypoxanthine guanine phosphoribosyl transferase, ribosomal protein L13a, lactate dehydrogenase A, and β-actin were used. Each replicate cycle threshold (CT) was normalized to the average CT of five endogenous controls on a per plate basis. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold-change in gene expression from LPS-stimulated versus saline (control) for each dietary group [30]. The genes differentially expressed were identified using a two-tailed, Student's *t*-test. The selection criteria included a *p*-value less than 0.05 and a mean difference equal to or greater than a twofold change in expression levels after LPS treatment. The statistical calculation was based on the web-based program of RT2 Profiler PCR Array Data Analysis. Alterations in mRNA levels that fit the above criteria were considered to be up- or down-regulated.

2.8.2 Plasma cytokine assays

To correlate with splenic microarray inflammatory chemokine/cytokine expression data, we assessed representative cytokines, such as TNF-α, IFN-γ, IL-1β, and a chemokine MIP2 in the plasma using ELISA. Plasma was diluted and used in ELISA assays as per the manufacturer's instructions. ELISA kits for the measurement of rat TNF-α, IFN-γ, IL-1β were obtained from R&D Systems and the MIP2 from Invitrogen Corporation (Camarillo, CA). For all the ELISAs, the intraassay CV ranged from 2 to 9% and interassay CV ranged from 4 to 10%.

2.9 Statistical analyses

Student's *t*-tests were used to compare any deviation from the control-fed rats to the other dietary groups for specific measures including the following: circulating hormone, cytokine, chemokine, and creatinine levels. One-way analysis of variance (ANOVA) was used followed by pairwise multiple comparisons (Holm-Sidak method using SigmaStat version 3.5 software, San Jose, CA) to distinguish differences among dietary groups for gene transcription fold increase and inflammatory protein levels. In order to determine if the mRNA response and circulating protein levels of cytokines and chemokines were significantly associated (negatively or positively) with the circulating level of 25OHD and thus influenced by vitD status, nonlinear regression analyses were conducted (SAS.93®, SAS, Cary, NC). The level of total 25OHD was the continuous dependent variable and the analyses were corrected for the absence or presence of mushroom powder in the diet, since mushrooms without $vitD_2$ have been shown to significantly modify immune response in a number of rodent studies using in vitro stimulation of spleen cells and thus represent a confounding variable in the regression analyses [31–42]. For all the statistical analyses, significance was indicated at $p \leq 0.05$.

3 Results

3.1 Growth

The presence of mushroom with adequate, excess, or deficient levels of bioavailable vitD had no apparent effect on final body weight in growing female rats at 10 wk of the feeding study. Use of EDTA blood collection tubes precluded serum calcium analyses, so other measures were used to determine evidence of hypercalcemia and soft tissue calcification, as explained earlier [22]. In the rat, the kidneys are the most sensitive organ to impaired function from hypercalcemia and nephrocalcinosis, which explains why plasma creatinine was measured (Table 1). Lack of significant differences in plasma creatinine among the dietary groups suggests an absence of toxic effects on renal function from long-term mushroom consumption with or without vitD or other possible toxic effects from long-term high vitD intake.

3.2 Bioavailability of vitD from mushrooms

$VitD_2$ from UVB-exposed mushrooms is highly bioavailable as shown in Table 1. Rats fed 5.0% UVB-exposed mushroom diet had mean plasma 25OHD of 155.4 ± 12.8 ng/mL when fed a level of vitD that is 30 times the National Research Council (NRC) recommendation for vitD in the growing rat [24]. Circulating 25OHD levels greater than 150 ng/mL in humans are considered to be excessive, and values greater than 30.02 ± 2.29 ng/ml are usually considered vitD-sufficient [7]. Rats fed the NRC level for $vitD_3$ with no mushroom (control diet) showed a final group mean 25OHD level of 32 ± 11 ng/mL, which is within the normal human reference range

Table 1. Growth, hormone, renal function biomarkers, and circulating cytokine levels in response to LPS challenge

Dietary group mean vitD intake per day (IU)	Control 20 IU D_3/day	No vitD 0 IU D_3/day	5% Untreated mushroom 2.4 IU D_2/day	2.5% UVB mushroom 300 IU D_2/day	5% UVB mushroom 600 IU D_2/day
Final body weight (g (*n*))	259.8 ± 22.0 (20)	264.3 ± 22.3 (20)	261.3 ± 27.0 (20)	265.0 ± 18.3 (20)	263.5 ± 19.7 (20)
Plasma creatinine[b] (mg/dL (*n*))	0.59 ± 0.08 (10)	0.63 ± 0.06 (10)	0.69 ± 0.09 (9)	0.67 ± 0.08 (10)	0.57 ± 0.09 (9)
Plasma 25OHD (ng/mL (*n*))	30.02 ± 2.29 (20)	3.41 ± 0.96[d] (20)	3.52 ± 0.95[d] (20)	122.9 ± 13.08[d] (20)	155.4 ± 12.28[d] (20)
Plasma PTH (pg/mL (*n*))	84.5 ± 19.4 (20)	147.1 ± 43.6 (20)	144.8 ± 24.5 (20)	89.2 ± 27.6 (20)	62.9 ± 2.18 (20)
IL-1β[c]	851.4 ± 103.0	860 ± 116.9	1410.9 ± 98.9[d]	2135.4 ± 324.0[d]	789.1 ± 172.7
IFN-γ[c]	1789.1 ± 415.8	2407.8 ± 360.2	2955.9 ± 244.5	1691.9 ± 186.4	2061.3 ± 311.5
TNF-α[c]	267.1 ± 83.7	131.7 ± 32.2	95.5 ± 18.2[d]	31.3 ± 9.1[d]	43.4 ± 7.5[d]
MIP2[c]	3171.1 ± 506.6	2002.8 ± 354.5[d]	1562.2 ± 342.5[d]	1385 ± 191.2[d]	544.3 ± 148[d]

a) Data are presented as mean ± SD for body weight, plasma creatinine, 25OHD, and PTH levels.
b) Creatinine levels shown here from saline-challenged rats only due to interference of LPS treatment with measurement.
c) Data are presented as mean ± SEM ($n = 6$–10 LPS-treated rats/group).
d) Group mean value is significantly different from the control group fed 20 IU/day vitD$_3$ ($p \leq 0.05$).

(30–100 ng/mL). Plasma 25OHD concentrations below 10 ng/mL as shown for the vitD-deficient and 5.0% unexposed mushroom groups are in the vitD-deficient range. Rats fed with vitD-deficient diet showed higher PTH levels compared to the vitD replete groups, but this did not reach statistical significance due to large variation in measurement within each dietary group (Table 1). Group mean PTH level in rats consuming the vitD-deficient 5.0% unexposed mushroom diet was also elevated relative to the control-fed group.

Group mean levels of circulating cytokines (IFN-γ, TNF-α, and IL-1β) and the chemokine MIP2 are also shown in Table 1. Plasma TNF-α and MIP2 were significantly lower in rats fed diets with 2.5 and 5% UVB light exposed mushrooms compared to control diet fed rats; however, IL-1β was significantly higher in rats fed 5% unexposed and 2.5% UVB light exposed mushrooms, suggesting that the differential increase could be related to a mushroom component other than vitD. Plasma levels of IFN-γ were not changed by unexposed or light-exposed mushrooms.

3.3 Characterization of splenic cell subpopulations, mitogenic response, and cytokine production

The percentage of CD3$^+$, CD4$^+$, CD8$^+$, CD45$^+$, and CD25$^+$ did not differ with consumption of UVB-exposed mushrooms when compared to the control diet (data not shown). Functional assessment of splenic T cells with Con A and B cells with LPS showed no difference in the proliferative potential across all dietary groups and furthermore, dietary treatment had no impact on Con A mediated secretion of IFN-γ, TNF-α, or IL-2 (data not shown), suggesting that vitD$_2$ from UVB-exposed mushrooms or dietary mushrooms alone was insufficient to improve adaptive immune response relative to the control diet.

3.3 NK cell activity

At all tested effector:target ratios, NK cell activity was enhanced in the 5% UVB-exposed mushroom group compared to the control group, which suggests that the increase may be dependent on the level of bioavailable 25OHD (Fig. 1). Our findings suggest that higher levels of vitD$_2$ from UVB-exposed mushrooms may promote innate immunity through enhancement of NK cell activity.

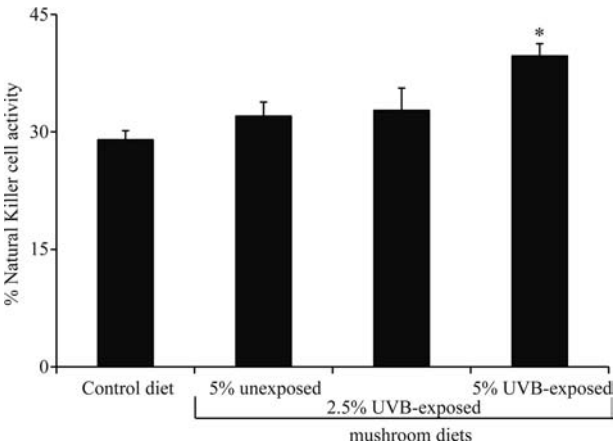

Figure 1. Splenic NK cell activity in rats fed unexposed control and UVB-exposed mushroom diets. Data are presented as % activity that was calculated as described in Materials and Methods. Each bar represents mean ± SEM, $n = 10$. *Group's mean value is significantly different from that of the control rats fed 20 IU vitD$_3$/day ($p \leq 0.05$).

Table 2. Effect of UVB-exposed mushroom on LPS-stimulated splenic inflammatory gene expression

Gene	Control	5% Unexposed mushroom	2.5% UVB-exposed mushroom	5% UVB-exposed mushroom
CCL2	75.14 ± 12.06	102.69 ± 15.38	30.68 ± 11.77[a)]	20.09 ± 7.34[a)]
CCL3	60.16 ± 7.32	60.06 ± 9.18	18.92 ± 2.80[a)]	29.01 ± 6.32[a)]
CCL4	95.10 ± 9.61	125.48 ± 14.09	26.77 ± 2.02[a)]	63.0 ± 10.46[a)]
CCL7	62.88 ± 8.44	104.28 ± 11.44[a)]	13.34 ± 2.07[a)]	16.45 ± 3.97[a)]
CCL9	70.36 ± 10.44	60.09 ± 10.04	13.94 ± 2.32[a)]	23.62 ± 6.02[a)]
CXCL1	877.10 ± 149.74	1083.45 ± 133.47	515.9 ± 229.72	199.08 ± 82.27[a)]
CXCL10	186.09 ± 29.91	91.47 ± 28.92[a)]	12.0 ± 3.27[a)]	5.0 ± 0.85[a)]
MIP2	1189.03 ± 293.03	2257.19 ± 517.8	806.77 ± 280.86	697.73 ± 142.28
IFN-γ	211.23 ± 31.13	93.67 ± 28.89[a)]	74.07 ± 28.96[a)]	23.64 ± 8.54[a)]
TNF-α	13.84 ± 3.10	7.96 ± 1.56	3.75 ± 0.47[a)]	3.01 ± 0.68[a)]
IL-1α	112.57 ± 23.24	90.74 ± 9.45	11.95 ± 1.13[a)]	35.68 ± 5.05[a)]
IL-1β	11.77 ± 1.77	9.48 ± 1.15	2.89 ± 0.81[a)]	5.04 ± 0.7[a)]

Data are presented as dietary group mean ± SEM ($n = 6$–10 rats/group). Gene expression data represent LPS-induced regulation for each gene as fold change compared to their corresponding saline controls.
a) Groups mean value is significantly different from the group mean value of the control rats fed 20 IU vitD$_3$/day ($p \leq 0.05$).

3.4 Gene expression and serum protein analysis

From the panel of 84 genes related to chemokine and chemokine receptors, ILs and their receptors, and cytokines, only 12 splenic genes were differentially expressed following LPS stimulation. UVB-exposed mushroom in the diet caused significant reduction in chemokines (Ccl2, Ccl3, Ccl4, Ccl7, Ccl9, Cxcl1, Cxcl10, and MIP2), cytokines (IFN-γ, TNF-α, IL-1α, and IL-1β) in response to LPS stimulation (Table 2). Figures 2, and 3A and B show the representative analytes (TNF-α, IL-1β, MIP2, and IFN-γ) in LPS-stimulated rat spleens from each of four dietary groups for both differential gene expression and plasma protein measures, respectively. To better visualize the influence of vitD status of the individual rats on immune response to acute LPS challenge, the fold increase in gene expression for each LPS-challenged rat was plotted against its plasma 25OHD level (Fig. 2). In addition, regression analyses of log-transformed differential gene fold expression measures and log-transformed plasma protein levels were conducted using plasma total 25OHD as the continuous dependent variable and the presence/absence of mushroom in the diet as a covariate. Predicted changes from these regression analyses are plotted in Fig. 3A (gene expression) and 3B (plasma proteins). The results of the regression analyses predicting change in gene expression (Fig. 3A) indicate an association of mushroom vitD$_2$ content and the

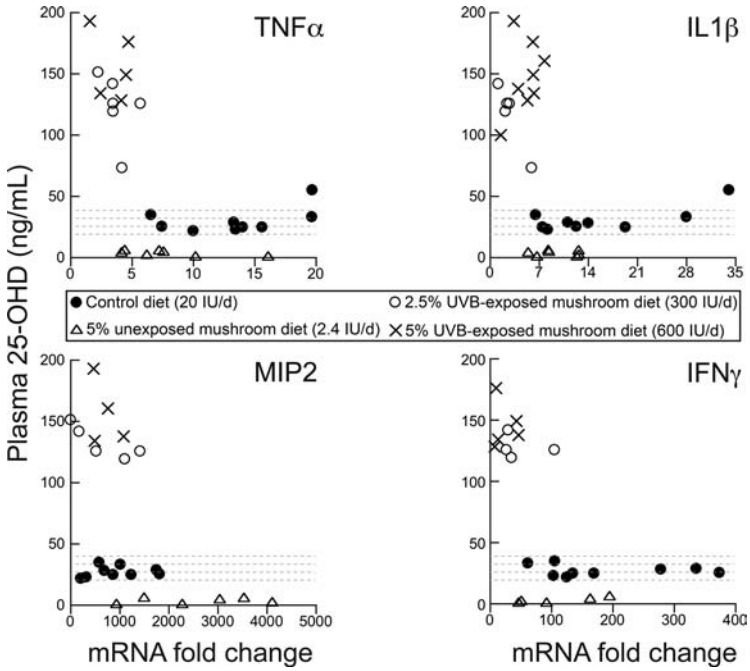

Figure 2. Scatter plot showing association of plasma vitD status with splenic inflammatory gene expression in rats. The four graphs are scatter plots showing the association between plasma total 25OHD levels (*y*-axis) and fold changes in mRNA levels (*x*-axis) of TNF-α, IL1β, MIP2, and IFN-γ following LPS challenge relative to mRNA levels after saline challenge. Individual rats fed control diet, consuming 20 IU vitD$_3$/day, are shown by the solid circle (●); rats consuming the 5% unexposed mushroom diet, consuming 2.4 IU vitD$_2$/day, are shown by the open triangle (Δ), those rats fed 2.5% UVB-exposed mushroom diet are represented by the open circle (○), and those fed 5% UVB-exposed mushroom diet are shown by the letter x (X), and they consumed 300 and 600 IU vitD$_2$/day, respectively. The stippled areas parallel to the *x*-axis in each graph represent the usual range of plasma total 25OHD observed in most adult men and women with adequate vitD status.

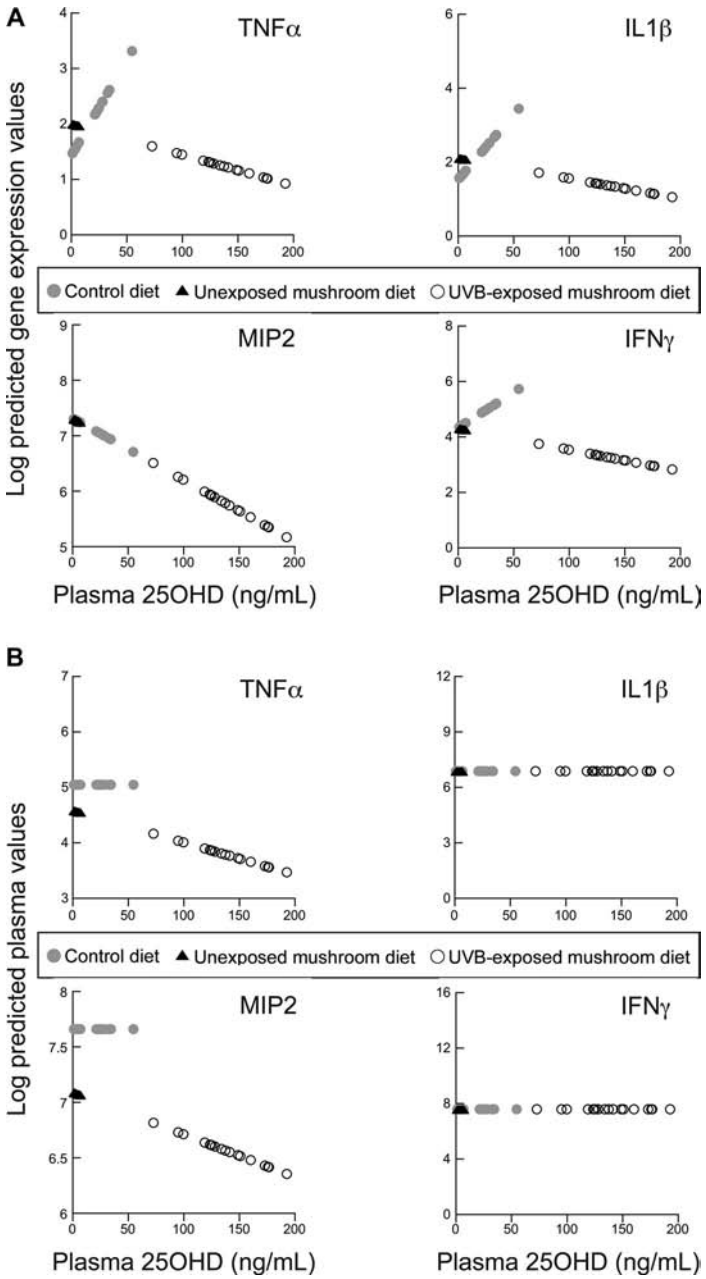

Figure 3. (A) Regression analyses showing association of log-transformed predicted fold gene expression values with plasma 25OHD levels in rats fed control diet (●), unexposed mushroom diet (▲), and UVB-exposed mushroom diet (○). (B) Regression analyses showing association of log-transformed predicted plasma cytokine protein levels with plasma 25OHD levels in rats fed with varying dietary groups. In both (A) and (B), four graphs plot the plasma total 25OHD level ($25OHD_2 + 25OHD_3$) on the *x*-axis against the log-transformed predicted fold gene expression (mRNA) level on the *y*-axis for Fig. 3A, and against the log-transformed predicted plasma cytokine protein levels on the *y*-axis for Fig. 3B. For both (A) and (B), the continuous variable regression analyses examined individual rat values from three dietary groups: (●, $n = 10$) represents control diet fed rats consuming diets without mushroom providing 20 IU $vitD_3$/day (absence of mushroom); (▲, $n = 10$) represents rats fed unexposed mushroom diets providing 2.4 IU $vitD_2$/day (presence of mushrooms); and (○, $n = 20$) represents rats fed the 2.5 and 5% UVB-exposed mushroom diets providing 300 and 600 IU $vitD_2$/day (presence of mushroom), respectively. The presence or absence of mushrooms were confounding variables in each figure.

suppression of TNF-α, IFN-γ, and IL-1β gene expression with every unit (nanograms per milliliters) 25OHD increase (Table 3). In the absence of mushrooms, TNF-α, IFN-γ, and IL-1β gene expression significantly increased with each nanograms per milliliters increase in total 25OHD. Independent of a mushroom effect, MIP2 gene expression was predicted to decrease with increases in 25OHD. Figure 3B shows a predicted significant decrease only with the circulating levels of TNF-α and MIP2 protein with increasing levels of total 25OHD.

In Fig. 2, control diet fed rats consuming an estimated 20 IU $vitD_3$/day with serum total 25OHD levels comparable to humans (~30 ng/mL) show limited suppression of the proinflammatory cytokine mRNA except for MIP2 (stippled box) compared to rats fed the 2.5 and 5% UVB-exposed mushrooms with total circulating 25OHD levels considered in the optimal range and higher for humans (>100 ng/mL) [7]. We also observed that rats fed vitD-deficient diets without mushroom had significantly lower expression of inflammatory mediators in the spleen (data not shown). This could be attributed to limited availability of precursor (25OHD) to spleen cells required for the synthesis of the active form of vitD. The hormonal or activated form of vitD (1, 25-dihydroxyvitamin D) binds to the vitD receptor

Table 3. Results of regression analyses: Predicted changes in gene expression and plasma protein levels of inflammatory cytokines associated with plasma 25OHD levels in the presence or absence of mushrooms

Biomarker expression	Dietary condition			*p*-Value
	Absence of Mushroom	Presence of mushroom	VitD effect only	
	Change in predicted value per nanograms per milliliters increase in 25OHD			
TNF-α gene expression	Increase by 0.038	Decrease by 0.0048		<0.0001
MIP2 gene expression	No change	No change	Decrease by 0.0067	0.0096
IFN-γ gene expression	Increase by 0.026	Decrease by 0.0077		0.0011
IL-1β gene expression	Increase by 0.035	Decrease by 0.0047		<0.0001
TNF-α protein	No change	Decrease by 0.00445		0.0009
MIP2 protein	No change	Decrease by 0.00181		0.0166
IFN-γ protein	No change	No change		non significant
IL-1β protein	No change	No change		non significant

activating the transcriptional complex needed to initiate gene transcription.

4 Discussion

Our 10-wk feeding protocol followed by an acute immunochallenge in growing rats not only demonstrated that UVB-exposed mushrooms are an effective source of vitD$_2$, but their consumption can also modulate immune response. For the first time, we present evidence that elevated levels of plasma 25OHD from UVB light-exposed mushroom consumption were significantly associated with reduced levels of inflammatory mediators, effectively suppressing the inflammatory response to endotoxin challenge in rats. UVB light exposed mushrooms, thus, have the potential to serve as an important functional food in providing both a much-needed rich food source of vitD and an effective modulator of immune response.

Many in vitro and in vivo studies exploring different varieties of edible mushrooms have demonstrated the immune-stimulating effects of mushroom consumption without high levels of vitD$_2$ [32–43]. Edible oyster mushroom extracts have demonstrated anti-inflammatory activity in RAW264.7 murine macrophages and in mice exposed to LPS [32]. Oyster mushroom concentrate reduced TNF-α, IL-6, IL-12, prostaglandin E$_2$, and nitric oxide secretion (downregulation of COX-2 and iNOS, respectively), which was shown to be mediated through the inhibition of activator protein-1 and nuclear factor-kappa B (NF-κB) transactivation. These in vitro anti-inflammatory effects were further confirmed in an in vivo LPS mouse model by marked reduction in circulating levels of TNF-α and IL-6 by intragastric gavage with oyster mushroom concentrate [32]. Similarly, white button mushroom consumption offered protection against colonic injury in mice treated with dextran sodium sulfate, which was partially attributed to increased TNF-α production locally and systemically [33]. A nonsignificant reduction in collagen-induced arthritis index was shown in mice fed 5% white button or shiitake mushrooms for 6 wk [34].

White button mushroom consumption has led to differential impacts on innate and adaptive immunity in rodent models depending on their age and health status. Feeding white button mushrooms to young or old mice had no impact on adaptive immunity as measured by Con A and PHA-mediated spleen cell proliferation or their ability to fight influenza virus [35, 36]. Conversely, a higher concentration of white button mushrooms (10% of diet) resulted in increased innate immunity as measured by NK cell activity in older mice [36, 37], and white button mushroom extract caused a dose-dependent maturation and increased antigen presentation by bone marrow derived dendritic cells [38]. In contrast to these earlier studies, our study showed that feeding white button mushrooms without vitD enrichment to young rats had no impact on innate or adaptive immune responses, though it is possible that mushrooms added to our diet at 5% of dry weight did not provide enough bioactive components to have an immunostimulatory effect.

A number of bioactive compounds common to different mushroom varieties have been shown to have immunostimulating properties, including β-glucan, α-glucan, chitosan, and phenolic compounds [43]. White button mushroom consumption has been shown to have beneficial health effects in both rodents and humans. Their consumption was associated with antiproliferative and prostate tumor inhibition in athymic mice [39], lower blood pressure and cholesterol in diabetic and hypercholestrolemic rats [40], and raises nitric oxide in mouse bone marrow derived macrophages [41]. Immune defense mechanisms associated with white button mushroom consumption in humans include an acceleration of salivary immunoglobulin A production [42], and the promotion of several hypocholesterolemic actions in healthy adults, which are associated with reduced risk of cardiovascular disease [43]. VitD status has also been inversely linked to cardiovascular disease and cancer [44], but available evidence is currently considered too weak to set dietary guidelines for adequate vitD intake aimed at prevention of any disease other than skeletal [45]. Nevertheless, the important role of vitD in immune response is well established in both humans and animal models [1, 2, 46–48].

There are limitations in extrapolating our findings in rats to humans. These limitations relate to the form of vitD tested ($vitD_2$) and the inherent species differences in innate immune defense between mice and men. Evidence suggests that $vitD_2$ is less effective than $vitD_3$ in raising plasma 25OHD levels in humans [49]. Then again, more recent evidence shows little difference between D_2 and D_3 in this regard when consumed daily [50]. Rats and humans differ in their hepatic metabolism of $vitD_2$ and D_3 with rodents preferentially hydroxylating $vitD_2$ over $vitD_3$ in contrast to humans and other species [51]. In this context, the rat may be more sensitive to the potential toxicity of $vitD_2$ in very high doses such as the intakes tested in this study. As reported earlier [22], despite very high plasma 25OHD levels, we found no evidence of vitD toxicity after 10 wk of feeding and examining toxic endpoints such as growth retardation, impaired renal function (elevated plasma creatinine), or soft tissue calcification (kidney, spleen, liver). Our findings of the safety of very high doses of $vitD_2$ from UVB-exposed mushroom consumption in the rat are consistent with those in Old World monkeys and humans consuming high levels of $vitD_2$ over months [52–54]. Recent studies in adults with poor vitD status [55] or limited sun exposure and no other dietary source of vitD [56] showed good bioavailability of vitD from mushrooms. These findings help to assure the appropriateness of extrapolating the bioavailability of mushroom $vitD_2$ in rats to the human situation.

Another limitation to the extrapolation of our findings in rats to humans concerns the species differences in vitD mechanism of action in immune defense. Rodents are unable to mediate a vitD-dependent innate antimicrobial protein response (cathelicidin and β-defensin peptides) in epithelial barrier tissues of the lung, gastrointestinal, and urinary/reproductive tracts, since unlike humans, rat genes in epithelial barrier tissues lack vitD response elements [57, 58]. Epigenetic chromatin alterations by plasma 25OHD levels are important in the regulation of innate and adaptive immunity in both humans and rodents. On the other hand, there is consistency in vitD-mediated adaptive immune response across humans and rodent models [59]. This subtle difference in innate immune mechanisms should be taken into consideration when applying the most compelling finding from our rat study to humans. We found that the moderate levels of circulating 25OHD in the control-fed rats, which are currently deemed adequate vitD status in humans, were not effective in quelling the inflammatory response to an intraperitoneal dose of LPS in the rat, as evidenced by expression and synthesis of inflammatory biomarkers. At the very least, this should cause us to question whether or not optimal immune function in healthy humans requires higher circulating levels of total 25OHD than currently considered to be in the normal range by the IOM (30–100 ng/mL) [45]. It remains to be determined whether or not modest levels of plasma 25OHD are associated with effective suppression of the human inflammatory response to inhaled or ingested microbes or endotoxins, the important first line of immune defense.

In summary, we used a growing rat model challenged with a sublethal dose of endotoxin after a prolonged feeding period to test the safety and efficacy of a new ingredient or postharvest treated food, which is considered an appropriate approach by others to test UVB-exposed food ingredients [60]. We demonstrated the potential for UVB-exposed white button mushrooms to provide a safe, bioavailable source of $vitD_2$, which when fed at higher levels was associated with a beneficial anti-inflammatory effect in LPS-challenged rats. In addition to their $vitD_2$ content, UVB-exposed white button mushrooms are a rich source of essential micronutrients and protein, along with other nonnutrient bioactive compounds such as β-glucan that has been shown to lower cardiovascular risk factors in humans [43]. Most recently, white button mushrooms (1 g/100 g) by themselves, and not $vitD_2$ enhanced by UVB light, fed to mice inoculated with *Citrobacter rodentium* caused a change in the composition of the normal colon flora and the urinary metabolome of mice [61], changes in the microbiome that resulted in better control of the inflammation and resolution of infection with *C. rodentium*.

In conclusion, one can say that from our perspective, but not necessarily that of the FDA, these UVB-treated mushrooms meet the globally accepted definition of a "functional food." Our findings add to the growing evidence that consumption of white button mushrooms, both UVB light exposed with $vitD_2$ and unexposed without enhanced $vitD_2$, "beneficially affects one or more target function in the body, beyond nutritional effects, in a way that is relevant to either an improved state of health and wellbeing and/or a reduction in the risk of disease," which is the definition of a functional food [43, 60–62]. UVB light exposed white button mushrooms and other varieties of edible fungi merit long-term clinical study to determine the full extent of their health benefits.

The authors thank the Mushroom Council and US Food and Drug Administration Center for Food Safety and Applied Nutrition for support. Kannan V. Balan was funded by the FDA Commissioner's Fellowship Program and Oak Ridge Institute for Science and Education (ORISE).

The findings and conclusions in this paper are those of the authors and do not necessarily represent the views and opinions of the US Food and Drug Administration.

The authors have declared no conflict of interest.

5 References

[1] Ralph, A. P., Lucas, R. M., Norval, M., Vitamin D and solar ultraviolet radiation in the risk and treatment of tuberculosis. *Lancet Infect. Dis.* 2013, *13*, 77–88.

[2] Schwalfenberg, G. K., A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol. Nutr. Food Res.* 2011, *55*, 96–108.

[3] Whiting, S., Greene, T., Calvo, M., Vitamin D intakes in North America and Asia-Pacific countries are not sufficient to prevent vitamin D insufficiency. *J. Steroid Biochem. Mol. Biol.* 2007, *103*, 626–630.

[4] Looker, A., Pfeiffer, C., Lacher, D., Schleicher, R. et al., Serum 25-hydroxyvitamin D status of the US population:1988–1994 compared to 2000–2004. *Am. J. Clin. Nutr.* 2008, *88*, 1519–1527.

[5] Bailey, R., Dodd, K., Goldman, J., Gache, J. et al., Estimation of total usual calcium and vitamin D intakes in the United States. *J. Nutr.* 2010, *140*, 817–822.

[6] Whiting, S., Langlois, K., Vantanparast, H., Greene-Finestone, L., The vitamin D status of Canadians relative to the 2011 Dietary Reference Intakes: an examination in children and adults with and without supplement use. *Am. J. Clin. Nutr.* 2011, *94*, 128–135.

[7] Holick, M., Binkley, N., Bischoff-Ferrari, H., Gordon, C. et al., Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical Practice Guideline. *J. Clin. Endocrinol. Metab.* 2011, *96*, 1911–1930.

[8] Calvo, M. S., Whiting, S. J., Barton, C. N., Vitamin D fortification in the US and Canada: current status and research needs. *Am. J. Clin. Nutr.* 2004, *80*, 1710S–17106S.

[9] Calvo, M. S., Whiting, S. J., Survey of current vitamin D fortification practices in the United States and Canada. *J. Steroid Biochem. Mol. Biol.* 2012, *136*, 211–213.

[10] Dietary Guidelines Advisory Committee, *The Report of the Dietary Guidelines Advisory Committee on the Dietary Guidelines for Americans, 2010*. Department of Health and Human Services, Washington, DC. Available from: http://www.cnpp.USDA.gov/dgas2010-dgacreport.htm. Accessed August 11, 2011.

[11] Mau, J., Chen, P., Yanf, J., Ultraviolet irradiation increased vitamin D2 content of edible mushrooms. *J. Agric. Food Chem.* 1998, *46*, 5269–5272.

[12] Phillips, K. M., Ruggio, D. M., Horst, R. L., Minor, B. et al., Vitamin D and sterol composition of 10 types of mushrooms from retail suppliers in the United States. *J Agric. Food Chem.* 2011, *59*, 7841–7853.

[13] Simon, R., Phillips, K., Horst, R., Munro, I., Vitamin D mushrooms: comparison of the composition of button mushrooms (Agaricus bisporus) treated post harvest with UVB light or sunlight. *J. Agric. Food Chem.* 2011, *59*, 8724–8732.

[14] Kalaras, M. D., Beelman, R. B., Elias, R. J., Effects of postharvest pulsed UV light treatment of white button mushrooms (Agaricus bisporus) on vitamin D2 content and quality attributes. *J. Agric. Food Chem.* 2012, *60*, 220–225.

[15] Whiting, S., Calvo, M., in: Feldman, D., Pike, W. J., Adams, J. S. (Eds.), *Vitamin D*, Wiley-Blackwell, San Diego, CA, 2011, pp. 979–1007.

[16] Calvo, M., Whiting, S., Barton, C., Vitamin D intake: a global perspective of current status. *J. Nutr.* 2005, *135*, 310–316.

[17] Hollis, B., Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D. *J. Nutr.* 2005, *135*, 317–322.

[18] Calvo, M., Whiting, S., in: Holick, M. (Ed.) *Vitamin D: Physiology, Molecular Biology and Clinical Applications*, Humana Press, Totowa, NJ, 2010, pp. 361–382.

[19] Mattila, P., Piironen, V., Uusi-Rauva, E., Koivistoinen, P., Vitamin D contents in edible mushrooms. *J. Agric. Food Chem.* 1994, *42*, 2449–2453.

[20] Roberts, J., Teichert, A., Hugh, T., Vitamin D2 formation from post-harvest UV-B treatment of mushrooms (Agaricus bisporus) and retention during storage. *J. Agric. Food Chem.* 2008, *56*, 4541–4544.

[21] Koyyalamudi, S., Jeong, S., Song, C., Cho, K., Pang, G., Vitamin D2 formation and bioavailability from Agaricus bisporus button mushroom treated with ultraviolet irradiation. *J. Agric. Food Chem.* 2009, *57*, 3351–3355.

[22] Calvo, M. S., Babu, U. S., Garthoff, L. H., Woods, T. O. et al., Vitamin D2 from light exposed mushrooms is safe, bioavailable and effectively supports bone growth in rats. *Osteoporos. Int.* 2013, *24*, 197–207.

[23] Mattila, P., Lampi, A.-M., Ronkainen, R., Tovio, J. et al., Sterol and vitamin D2 contents in some wild and cultivated mushrooms. *Food Chem.* 2002, *76*, 293–298.

[24] Fleet, J., Gliniak, C., Xue, Y., Smith, K. et al., 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.

[25] Garthoff, L., Sabotka, T., From farm to table to brain: foodborne pathogen infection and the potential role of the neuro-immune-endocrine system in neuro sequelae. *Nutr. Neurosci.* 2001, *4*, 333–374.

[26] Babu, U. S., Weisenfeld, P. W., Collins, T. F., Sprando, R. et al., Impact of high flaxseed diet on mitogen-induced proliferation, IL-2 production, cell subsets and fatty acid composition of spleen cells from pregnant and F1 generation Sprague-Dawley rats. *Food Chem. Toxicol.* 2003, *41*, 905–915.

[27] Lillehoj, H. S., Min, W., Babu, U. S., Burnside, J. et al., Molecular, cellular, and functional characterization of chicken cytokines homologous to mammalian IL-13 and Il-2. *Vet. Immunol. Immunopathol.* 2001, *82*, 229–244.

[28] Liao, W., Lin, J. X., Leonard, W. J., IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of t helper cell differentiation. *Curr. Opin. Immunol.* 2011, *23*, 598–604.

[29] Semaeva, E., Tenstad, O., Skavland, J., Iversen, P. O. et al., Access to the spleen microenvironment through lymph shows local cytokine production, increased cell flux, and altered signaling of immunse sells during lipopolysaccharide-induced acute inflammation. *J. Immunol.* 2010, *184*, 4547–4556.

[30] Scmittgen, T. D., Livak, K. J., Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 2008, *3*, 1101–1108.

[31] Borchers, A. T., Krishnamurthy, A., Keen, K. L., Meyers, F. J., Gershwin, E. G., The immunobiology of mushroom. *Exp. Biol. Med.* 2008, *233*, 259–276.

[32] Jedinak, A., Dudhgaonkar, S., Wu, Q. L., Simon, J. et al., Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF-kappaB and AP-1 signaling. *Nutr. J.* 2011, *10*, 52–61.

[33] Yu, S., Weaver, V., Martin, K., Cantorna, M. T., The effects of whole mushrooms during inflammation. *BMC Immunol.* 2009, *10*(12), 1–13.

[34] Chandra, L., Alexander, T., Traore, D., Lucas, E. A. et al., White button and shitake mushrooms reduce the incidence and severity of collagen-induced arthritis in dilute brown non-agouti mice. *J. Nutr.* 2011, *141*, 131–136.

[35] Xu, Y., Na, L., Ren, Z., Xu, J. et al., Effect of dietary supplementation with white button mushrooms on host resistance to influenza infection and immune function in mice. *Br. J. Nutr.* 2013, *109*, 1052–1061.

[36] Wu, D., Han, S. N., Bronson, R. T., Smith, D. E. et al., Dietary supplementation with mushroom-derived protein bound glucan does not enhance immune function in young and old mice. *J. Nutr.* 1998, *128*, 193–197.

[37] Wu, D., Pae, M., Ren, Z., Guo, Z. et al., Dietary supplementations with white button mushroom enhances natural killer cell activity in C57BL/6mice. *J. Nutr.* 2007, *137*, 1472–1477.

[38] Ren, Z., Guo, Z., Meydani, S. N., Wu, D., White button mushroom enhances maturation of bone marrow-derived dendritic cells and their antigen presenting function in mice. *J. Nutr.* 2008, *138*, 544–550.

[39] Adams, L. S., Phung, S., Wu, X., Ki, L. et al., White button mushrooms (Agaricus bisporus) exhibit anti proliferative and proapoptotic properties and inhibits prostate tumor growth in athymic mice. *Nutr. Cancer* 2008, *60*, 744–756.

[40] Jeong, S. C., Jeong, Y. T., Yang, Y. K., Islam, R. et al., White button mushroom (Agaricus bisporus) lowers blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats. *Nutr. Res.* 2010, *30*, 49–56.

[41] Volman, J. J., Helsper, J. P. F. G., Wei, S., Baars, J. J. et al., Effects of mushroom-derived β-glucan-rich polysaccharide extracts on nitric oxide production by bone marrow-derived macrophages and nuclear factor-κB transactivation in Caco-2 reporter cells: can effects be explained by structure? *Mol. Nutr. Food Res.* 2010, *54*, 268–276.

[42] Jeong, S. C., Koyyalamudi, S. R., Panf, G., Dietary intake of Agaricus bisporus white button mushroom accelerates salivary immunoglobulin A secretion in healthy volunteers. *Nutrition* 2012, *28*, 527–531.

[43] Guillamon, Y., Garcia-Lafuente, A., Lozano, M., D'Arrigo, M. et al., Edible mushrooms: role in the prevention of cardiovascular diseases. *Fitoterapia* 2010, *81*, 715–723.

[44] Wei, M. Y., Giovannucci, E. L., Vitamin D and multiple health outcomes in the Havard cohorts. *Mol. Nutr. Food. Res.* 2010, *54*, 1114–1126.

[45] Institute of Medicine, Food and Nutrition Board, *Dietary Reference Intakes for Calcium and Vitamin D; National Academy of Science (NAS), 2011*, The National Academy Press, Washington, DC, 2011.

[46] White, J. H., Vitamin D metabolism and signaling in the immune system. *Rev. Endocr. Disord.* 2012, *13*, 21–29.

[47] Liu, P. T., Stenger, S., Li, H., Wenzel, L. et al., Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006, *311*, 1770–1773.

[48] Hewison, M., Vitamin D and the immune system: new perspectives on an old theme. *Rheum. Dis. Clin. North Am.* 2012, *38*, 125–139.

[49] Trikovic, L., Lambert, H., Hart, K., Smith, C. P. et al., Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am. J. Clin. Nutr.* 2012, *95*, 1357–1364.

[50] Biancuzzo, R. M., Clarke, N., Reitz, R. E., Travison, T. G., Holick, M. F., Serum concentrations of 1,25-dihydroxyvitamin D3 in response to vitamin D2 and vitamin D3 supplementation. *J. Clin. Endocrinol. Metab.* 2013, *98*, 973–979.

[51] Horst, R., Napoli, J., Littledike, T., Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat and chick. *Biochem. J.* 1982, *204*, 185–189.

[52] Hunt, R., Garcia, F., Walsh, R., A comparison of the toxicity of ergocalciferol and cholecalciferol in rhesus monkeys. *J. Nutr.* 1972, *102*, 975–986.

[53] Stephenson, D., Peiris, A., The lack of vitamin toxicity and megadose of daily ergocalciferol (D2) therapy: a case report and literature review. *South. Med. J.* 2009, *102*, 765–768.

[54] Binkley, N., Gemar, D., Engelke, J., Gangnon, J. et al., Evaluation of ergocalciferol of cholecalciferol dosing, 1,6000 IU daily or 50,000 IU monthly in older adults. *J. Clin. Endocrinol. Metab.* 2011, *96*, 981–988.

[55] Ozzard, A., Hear, G., Morrison, G., Hoskin, M., Vitamin D deficiency treated by consuming UVB-irradiated mushrooms. *Br. J. Gen. Pract.* 2009, *58*, 644–645.

[56] Urbain, P., Singler, F., Ihorst, G., Biesalski, H.-K. et al., Bioavailability of vitamin D2 from UV-B-irradiated button mushrooms in healthy adults deficient in serum 25-hydroxyvitamin D: a randomized controlled trial. *Euro. J. Clin. Nutr.* 2011, *65*, 965–971.

[57] Gombart, A. F., The vitamin D-antimicrobial peptide pathway and its role in protection against infection. *Future Microbiol.* 2009, *4*, 1151–1165.

[58] Liu, P. T., Schenk, M., Walker, V. P., Dempsey, P. W. et al., Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS One* 2009, *4*, e5810.

[59] Chambers, E. S., Hawrylowicz, C. M., The impact of vitamin D on regulatory T cells. *Curr. Allergy Asthma Rep.* 2011, *11*, 29–36.

[60] Simons, R. R., Borzelleca, J. F., DeLuca, H. F., Weaver, C. M., Safety assessment of the post-harvest treatment of button mushrooms (Agaricus bisporus) using ultraviolet light. *Food Chem. Toxicol.* 2013, *56*, 278–289.

[61] Varshney, J., Ooi, J. H., Jayarao, B. M., Albert, I. et al., White button mushrooms increase microbial diversity and accelerate the resolution of Citerobacter rodentium infection in mice. *J. Nutr.* 2013, *143*, 525–532.

[62] Hasler, C. M., Brown, A. C., Position of the American Dietetic Association: functional foods. *J. Am. Diet. Assoc.* 2009, *109*, 735–746.