Original Communication

Microalgae-derived sterols do not reduce the bioavailability of oral vitamin D_3 in mice

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Abstract: Microalgae have drawn increasing attention as sustainable food sources, also because of their lipid-lowering phytosterols. As phytosterols are also discussed critically regarding their effect on the availability of fat-soluble vitamins, this study aimed to investigate microalgae-derived phytosterols and their effect on vitamin D status. GC–MS analysis showed large variations in the phytosterol profiles of microalgal species. The most frequent sterols were β-sitosterol and stigmasterol. To investigate their effects on vitamin D status, 40 mice were randomized to four groups and fed a vitamin D_3-adequate (25 μg/kg) Western-style diet with 0% phytosterols (control) or 1% ergosterol (a fungal sterol not typical for microalgae), β-sitosterol or stigmasterol for four weeks. Contrary to the hypothesis that phytosterols adversely affect vitamin D uptake, mice fed β-sitosterol had significantly higher concentrations of vitamin D_3 in plasma (3.15-fold, $p<0.01$), liver (3.15-fold, $p<0.05$), and skin (4.12-fold, $p<0.005$) than the control group. Small increases in vitamin D_3 in plasma and skin were also observed in mice fed stigmasterol. In contrast, vitamin D_3 levels in the ergosterol and control groups did not differ. The increased tissue levels of vitamin D_3 in mice fed β-sitosterol and stigmasterol were not attributable to the observed reduction in liver triglycerides in these groups. The data rather suggest that changes in bile acid profiles were responsible for the beneficial effect of microalgae sterols on the bioavailability of vitamin D_3. In conclusion, consumption of microalgae might not adversely affect vitamin D status.

Keywords: Microalgae, β-sitosterol, stigmasterol, ergosterol, vitamin D_3, mice

Introduction

Microalgae are a heterogeneous group of unicellular, photoautotrophic or heterotrophic microorganisms that have the potential as sustainable sources to meet future global needs for essential nutrients and provide healthy diet substances. Several microalgae contain relatively high quantities of long-chain n-3 fatty acids [1, 2] and high-quality proteins [3] as well as vitamins, carotenoids and other pigments [4, 5]. Microalgae can be cultivated under controlled conditions (e.g., temperature, pH, light, salinity, inorganic substances) to enable tailored nutrient profiles [6, 7]. In addition, microalgae are capable of producing large quantities of highly diverse phytosterols [8, 9, 10] that can lower plasma cholesterol [11] by (i) replacing cholesterol from micelles, (ii) competing with cholesterol for uptake into enterocytes or (iii) stimulating the reverse sterol transporters ATP binding cassette (ABC) G5 and G8 in the gut (Electronic Supplementary Material [ESM] 1, Figure E1) [12, 13].

In addition to the beneficial effects of phytosterols on circulating cholesterol, they are viewed critically regarding their potential adverse effect on the absorption of fat-soluble vitamins [14]. Among these, vitamin D is of particular interest because of the implemented policies of many countries to reduce the burden of vitamin D deficiency in vulnerable groups by the admittance of vitamin D-enriched foods or by the recommendation of vitamin D supplements [15, 16, 17]. Goncalves et al. were the first to show that β-sitosterol is capable of reducing the 2 h, 4 h and 7 h postprandial plasma vitamin D_3 concentrations in mice force-fed with vitamin D_3-rich emulsions [18]. These authors further found that phytosterols can significantly impair the incorporation of vitamin D_3 into mixed micelles [18].

Our own studies demonstrated that the reverse sterol transporters ABCG5/ABCG8 appear to also be involved in

reverse transport and subsequently in the reduced bioavailability of oral vitamin D_3 [19]. Conversely, data obtained from clinical trials are not indicative of any negative effect of phytosterols on the vitamin D status assessed by the plasma concentration of 25-hydroxyvitamin D (25(OH)D) [20, 21, 22, 23, 24, 25].

Because data on the types and quantities of sterols in microalgae are scarce, twelve microalgal species of different taxonomic groups that are potential novel candidates for human nutrition were analyzed regarding their phytosterol profiles. Selected microalgae-derived phytosterols were then used to investigate their long-term effect on vitamin D_3 storage and status in comparison to no phytosterols or fungal ergosterol in mice fed vitamin D_3-adequate Western-type diets. As phytosterols are capable of influencing lipid levels, vitamin D_3 data were correlated with lipid and bile acid data to ascertain whether changes in vitamin D_3 status are caused by phytosterol-induced changes in lipid metabolism.

Materials and methods

Cultivation and preparation of the microalgal species

The biomass of microalgal species was obtained from the Competence Center Algal Biotechnology (Department of Applied Biosciences and Process Technology, Anhalt University of Applied Sciences, Koethen, Germany). In total, 12 microalgal species of different taxonomic groups, including freshwater, marine, terrestrial, and aero-terrestrial organisms, were preselected on the basis of comprehensive literature research and cultivated under optimized conditions depending on literature recommendations or long-standing experience (ESM 1, Table E1). Five of these species were proprietary isolates from the Koethen Algae Strain Collection (KASC).

Microalgae were cultivated at room temperature either in 2 L bubble column photobioreactors or in 10 L cultivation flask photobioreactors. Commonly known culture media BBM, BG11, ES, Šetlik, SWES, OHM, f/2, and KUHL were composed as described elsewhere (e.g., www.epsag.uni-goettingen.de). The cultures were aerated with CO_2-enriched air (1.1 vvm, 1% CO_2, 1 L CO_2 h^{-1}) and illuminated from one side with cool white fluorescent light at 100–250 µmol m^{-2} s^{-1} (14:10 h light:dark period) provided by fluorescent bulbs (TL-D 36 W/865, Philips, Hamburg, Germany) arranged in horizontal rows of six. Growth behavior, cell density, and nutrient concentration were measured to determine the onset of the stationary phase (data not shown). Biomass was harvested during growth and/or stationary phase, separated either by centrifugation, and washed twice with deionized water. All samples were disintegrated in a laboratory agitator bead mill (MicroSerie, Netzsch, Selb, Germany) using zirconium oxide grinding beads (ZetaBeads® 0.3 mm, Netzsch) until complete disruption of the cells was observed by microscopy. The disintegrated biomass was lyophilized and stored at −80 °C until analysis.

Analysis of phytosterols in microalgal species

The analysis of sterols in microalgal species was performed according to method DGF-F-III 1 [26]. In brief, samples were spiked with betulin in acetone as an internal standard and saponified with ethanolic potassium hydroxide by boiling under reflux for 15 min. The hot solution was dissolved with ethanol and further applied to an aluminum oxide column (Merck, Darmstadt, Germany). The unsaponifiable matter with the sterols was eluted by ethanol followed by diethyl ester, while fatty acid anions were retained on the column. Then, the sterol fraction was separated from unsaponifiable matter by thin-layer chromatography (Merck) with a mixture of hexane/diethyl ether (1/2, v/v) as the flow agent. The zone with the sterols was identified by spraying the plate with methanol, and the marked zone was removed quantitatively from the plate by scratching off the silica completely using a spatula. Then, ethanol was added to the collected silica to ensure desorption of betulin and sterols, and the solution was extracted with diethyl ether, filtered into a flask and transferred to a reaction vial. The solvent was evaporated. Sterol trimethylsilyl ethers were prepared by adding 100 µL of silylation reagent (50 µL 1-methyl imidazole+1 mL N-methyl-N(trimethyl-silyl)-hepta-fluorobutyramide) to the isolated sterols. The vial was sealed and heated in an oven for 15 min at 102±3 °C. After cooling to room temperature, the solution was subjected to a gas chromatograph.

The compounds were separated on an SE 54 CB (Macherey-Nagel, Düren, Germany; 50 m, 0.32 mm ID, 0.25 µm film thickness). Further parameters were as follows: hydrogen as the carrier gas, split ratio 1:20, injection and detection temperature adjusted to 320 °C, temperature program: 245 °C–260 °C at 5 °C/min. Peaks were identified either by standard compounds (β-sitosterol, campesterol, stigmasterol), by a mixture of sterols isolated from rapeseed oil (brassicasterol) or by a mixture of sterols isolated from sunflower oil (7-stigmasterol and 7-campesterol). All other sterols were identified by GC–MS.

Experimental conditions of the mouse study

To investigate the effect of microalgae-specific phytosterols in comparison to fungal ergosterol or no phytosterol on vitamin D status, a mouse study was conducted. The experimental procedures described below followed the established guidelines for the care and handling of laboratory animals according to the US National Research Council [27] and were approved by the animal welfare committee of Martin Luther University Halle-Wittenberg (approval number: H1-4/T1-15). All mice were kept in pairs in a room controlled for temperature (22±2 °C), light (12-h light, 12-h dark cycle) and relative humidity (50–60%) and had free access to food and water.

Forty 4-week-old male C57BL/6J mice (Charles River, Sulzfeld, Germany) with an initial average body weight of 14.4±1.2 g were randomized to four treatment groups of 10 animals each and were fed diets with 0% phytosterol (control), 1% ergosterol (fungal sterol), 1% β-sitosterol, or 1% stigmasterol for four weeks. The basal Western-type diet consisted of (per kg) 387 g starch, 200 g sucrose, 200 g casein, 100 g lard, 50 g vitamin and mineral mixture, 50 g cellulose, 10 g cholesterol, and 3 g DL-methionine. The phytosterols were added to the diet in exchange for starch. Vitamins and minerals were added to the diet according to recommendations of the National Research Council [27]. Vitamin D was added to the diet as vitamin D_3 at a concentration of 25 µg/kg.

Individual body weights and feed intake (of two mice per cage) were recorded weekly. At the end of the dietary intervention, all mice were sacrificed after a 4-h food deprivation period. Blood was collected into heparin tubes (Sarstedt, Nümbrecht, Germany), and plasma was separated by centrifugation at 2000×*g* for 10 min at room temperature. The liver and skin samples were harvested, and fecal samples were collected from the rectum. All samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

Analysis of lipids in plasma and liver

Triglyceride and cholesterol concentrations were quantified in plasma and liver using enzymatic reagent kits according to the manufacturer's protocol (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). The preparation of liver extracts was performed as described elsewhere [28].

Analysis of vitamin D_3 metabolites in plasma and tissues

The concentrations of vitamin D_3 and 25(OH)D_3 were measured using LC–MS/MS as described previously [29]. In brief, sample aliquots were mixed with internal standards (vitamin D_3–d_7, Toronto Research Chemicals, Toronto, Canada; 25(OH)D_3–d_6, Chemaphor Chemical Services, Ottawa, Canada), and saponified with potassium hydroxide to extract the vitamin D_3 metabolites from the samples. Subsequently, vitamin D_3 metabolites were extracted with *n*-hexane and washed with ultrapure water. Tissue samples were further purified by preparative HPLC (1100 Series, Agilent Technologies, Waldbronn, Germany). All samples were then derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, Sigma Aldrich, Taufkirchen, Germany), dried and resolved in methanol and 10 mM ammonium formate (4/1, v/v). The samples were injected into the LC–MS/MS (1260 Infinity Series, Agilent Technologies; QTRAP 5500, SCIEX, Darmstadt, Germany). The system was operated in positive electrospray ionization mode. For quantification of vitamin D_3, a Hypersil ODS C18 column (120 A, 5 µm, 150×2.0 mm^2, VDS Optilab, Berlin, Germany) was used, and for quantification of 25(OH)D_3, a Poroshell C18 column (120 A, 2.7 µm, 50×4.6 mm^2, Agilent Technologies) was used. The quantifier mass transitions of the PTAD adducts were as follows: vitamin D_3 560>298, vitamin D_3–d_7 567>298, 25(OH)D_3 576>301, and 25(OH)D_3–d_6 582>298. All analytes were verified by specific qualifier mass transitions. Calibration curves were constructed with standard solutions (all from Sigma–Aldrich) by plotting the ratio of the analyte peak area to the internal standard peak area versus the concentration of the analytes.

Analysis of relative mRNA abundance

The relative mRNA abundance was analyzed by real-time RT–PCR. The isolation of total RNA, cDNA synthesis and the protocol for real-time RT–PCR are described in detail elsewhere [30]. The relative mRNA abundance of target genes was calculated by the modified ΔΔCT method of Pfaffl [31] using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), hypoxanthine guanine phosphoribosyltransferase (*Hprt*) and ribosomal protein, large, P0 (*Rplp0*) as reference genes. Primer pairs for *Hprt* (NM_013556), fatty acid synthase (*Fasn*, NM_007988), fibroblast growth factor 15 (*Fgf15*, NM_008003) and sterol-C5-desaturase (*Sc5d*, NM_172769) were purchased from Sigma–Aldrich (www.kicqstart-primers-sigmaaldrich.com). Sequences of the other primers used for PCR were as follows: *Gapdh* (XM_001473623) forward 5'-TGCACCACCAACTGCTTA-'3, reverse 5'-GGATGCAGGGATGATGTTC-'3; *Rplp0* (NM_007475) forward 5'-GAAACTGCTGCCTCACATCCG-'3, reverse 5'-CTGGCACAGTGACCTCACACG-'3; ATP binding cassette subfamily G member 5 (*Abcg5*, NM_031884) forward 5'-TGGATCCAACACCTCTATGCTAAA-'3, reverse 5'-GGCAGGTTTTCTCGATGAACTG-'3; ATP binding cassette subfamily G member 8 (*Abcg8*, NM_026180)

forward 5'-TGCCCACCTTCCACATGTC-'3, reverse 5'-ATGAAGCCGGCAGTAAGGTAGA-'3; acyl-coenzyme A oxidase 1 (*Acox1*, NM_015729) forward 5'-CAGGAAGAGCAAGGAAGTGG-'3, reverse 5'-CCTTTCTGGCTGATCCCATA-'3; CD36 molecule (*Cd36*, NM_001159558) forward 5'-GAGCAACTGGTGGATGGTTT-'3, reverse 5'-GCAGAATCAAGGGAGAGCAC-'3; carnitine palmitoyltransferase 1a (*Cpt1a*, NM_031559) forward 5'-CCAGGCTACAGTGGGACATT-'3, reverse 5'-GAACTTGCCCATGTCCTTGT-'3; NPC1 like intracellular cholesterol transporter 1 (*Npc1l1*, NM_207242) forward 5'-ATCCTCATCCTGGGCTTTGC-'3, reverse 5'-GCAAGGTGATCAGGAGGTTGA-'3; peroxisome proliferator activated receptor alpha (*Ppara*, NM_001113418) forward 5'-AGGCAGATGACCTGGAAAGTC-'3, reverse 5'-ATGCGTGAACTCCGTAGTGG-'3; scavenger receptor class B, member 1 (*Scarb1*, NM_016741) forward 5'-GTCCGCATAGACCCGAGCAG-'3, reverse 5'-CCAGCGCCAAGGTCATCATC-'3; and sterol regulatory element binding transcription factor 1 (*Srebf1*, NM_011480) forward 5'-GCACCCTCTTGCTCTGTAGG-'3, reverse 5'-ACCAAGCCAGCAAATACACC-'3 (Eurofins Genomics, Ebersberg, Germany).

Analysis of fecal bile acids

To determine the concentration of bile acids, feces of mice were collected from the rectum at the end of the study. Fecal samples were freeze-dried and sent to MS-Omics (Vedbaek, Denmark) for further preparation and analysis. In brief, the samples were extracted with methanol, and the extracts were centrifuged with tube filters for purification. The filtrate was injected into a Thermo Scientific Vanquish LC coupled to Thermo Q Exactive HF MS. Ionization was achieved by negative electrospray ionization. The chromatographic separation of bile acids was carried out on a Waters Acquity HSS T3 (1.8 µm, 2.1×150 mm^2). The column temperature was set at 30 °C. The mobile phases consisted of (A) 10 mM ammonium acetate and (B) methanol/acetonitrile (1/1, v/v). Bile acids were eluted by increasing the proportion of B in A from 45 to 100% for 16 min with a total flow rate of 0.3 mL/min. Peak areas were extracted using TraceFinder 4.1. The identification of compounds was based on the accurate mass and retention time of authentic standards.

Statistics

Values are expressed as the mean±standard error of mean (SEM) if not stated otherwise. Statistical analysis was performed with SPSS statistical software (version 25, IBM, Armonk, USA). All data were subjected to the Shapiro–Wilk normality test and Levene's test of homoscedasticity. If the data followed a normal distribution, one-way analysis of variance (ANOVA) was used to identify differences between the treatments. In the case of homoscedasticity, Tukey's test was applied as the suitable *post hoc* group comparison. In the case of heteroscedasticity, Welch's ANOVA was used followed by *post hoc* comparison via the Games Howell test. If the data were not normally distributed, differences between the groups were analyzed using the non-parametric Kruskal-Wallis test followed by a multiple group comparison via the Mann–Whitney U test with Bonferroni correction. To assess correlations between bile acids in feces and stores of vitamin D_3 in the liver and between lipids and vitamin D_3 in the liver and plasma, Spearman's rank correlation coefficient (Rho) was calculated. $P<0.05$ was considered to be significantly different.

Results

Phytosterol profiles of microalgae

The data show large variations in the types and concentrations of phytosterols in the microalgal species analyzed (Table 1). The highest phytosterol concentrations were found in *Myrmecia* sp. followed by *Nitzschia* sp., which contained 7.76 and 6.86 g, respectively, per 100 g of microalgae dry matter. The majority of the analyzed microalgal species had noticeable amounts of β-sitosterol (Table 1). The highest β-sitosterol concentration was found in *Nitzschia* sp., followed by *Tetradesmus* sp. and *Eustigmatos* sp., whereas it was not detectable in *Phaeodactylum* sp., *Nostoc* sp., *Crysotila* sp. and *Tetraselmis* sp. High concentrations of stigmasterol were particularly found in *Myrmecia sp.*, *Klebsormidium* sp., and *Spongiochloris* sp.; *Crysotila* sp. and *Nannochloropsis* sp. had medium concentrations of stigmasterol, while it was absent in *Tetradesmus* sp., *Chlorococcum* sp., *Nostoc* sp., *Nitzschia* sp., *Eustigmatos* sp., *Tetraselmis* sp. and *Phaeodactylum* sp. (Table 1). Many microalgal species also contained considerable amounts of campesterol, whereas brassicasterol, 24-methylencholesterol, campestanol, 7-campesterol, 5,23-stigmastadienol, 5-avenasterol, 7-stigmasterol and 7-campesterol were only found in a few microalgal species or in small quantities (Table 1). 7-Avenasterol, stigmasta-5,24-diene-3-ol, sitostanol and clerosterol were not detected in any of the microalgae analyzed (data not shown).

Impact of microalgae-specific phytosterols on vitamin D_3 and lipid metabolism

To investigate the role of microalgae-derived phytosterols on vitamin D_3 status, we used the quantitatively most important and commercially available phytosterols

Table 1. Phytosterol concentrations (mg/100 dry matter) in different algal species

Species	Phase	Brassicasterol $C_{28}\Delta^{5,22}$	24-Methylenecholesterol $C_{28}\Delta^{5,24}$	Campesterol $C_{28}\Delta^{5}$	Campestanol $C_{28}\Delta^{0}$	Stigmasterol $C_{29}\Delta^{5,22}$	7-Campesterol $C_{28}\Delta^{7}$	5,23-Stigmastadienol $C_{29}\Delta^{5,23}$	β-Sitosterol $C_{29}\Delta^{5}$	5-Avenasterol $C_{29}\Delta^{7,24(28)}$	7-Stigmasterol $C_{29}\Delta^{5,7,22}$	Sum
Tetradesmus sp.	SP	n.d.	n.d.	111	n.d.	n.d.	n.d.	17.9	2954	n.d.	219	3303
Spongiochloris sp.	SP	103	n.d.	608	n.d.	1267	n.d.	n.d.	84.6	n.d.	21	2083
Chlorococcum sp.	GP	54.1	n.d.	108	n.d.	n.d.	n.d.	n.d.	26.2	n.d.	n.d.	322
	SP	31.2	n.d.	188	n.d.	n.d.	n.d.	n.d.	48.2	n.d.	n.d.	269
Nannochloropsis sp.	GP	11.5	30.4	n.d.	n.d.	n.d.	58.6	55.8	1229	659	n.d.	2103
	SP	n.d.	n.d.	298	1373	60.0	n.d.	n.d.	17	n.d.	n.d.	1747
Nostoc sp.	SP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0
Nitzschia sp.	SP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	170	5094	1595	n.d.	6859
Eustigmatos sp.	SP	n.d.	n.d.	11.9	n.d.	n.d.	n.d.	n.d.	1874	n.d.	n.d.	1886
Klebsormidium sp.	SP	n.d.	n.d.	1062	n.d.	1463	n.d.	n.d.	366	n.d.	n.d.	2890
Tetraselmis sp.	SP	n.d.	118	3211	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3329
Crysotila sp.	SP	425	16.7	77.9	841	436	n.d.	n.d.	n.d.	n.d.	n.d.	1796
Myrmecia sp.	SP	104	116	1253	n.d.	4762	35.4	214	1241	n.d.	n.d.	7763
Phaeodactylum sp.	SP	799	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	799

GP: growth phase; SP: stationary phase; n.d.: not detectable.

β-sitosterol and stigmasterol and compared their effect with that of ergosterol or a diet containing no phytosterol.

Mice fed the diet containing adequate amounts of vitamin D_3 and 1% cholesterol without phytosterols (control) and mice fed the diet supplemented with 1% ergosterol, β-sitosterol, or stigmasterol did not differ in food intake or final body weight (Table 2). Unexpectedly, among the four groups, mice fed β-sitosterol showed by far the highest concentrations of vitamin D_3 in the liver, skin and plasma (Figure 1A–1C). Compared to the control group, stigmasterol-fed mice had moderately higher vitamin D_3 concentrations in skin and plasma but not in the liver. The effect of both microalgae-specific phytosterols on vitamin D_3 was not significantly different from that of ergosterol, although the vitamin D_3 levels in the liver and plasma of β-sitosterol-fed mice were more than twice as high as those in the ergosterol group (Figures 1A–1C). Despite marked differences in the plasma and tissue vitamin D_3 concentrations, the circulating level of 25(OH)D_3 did not differ between the four groups of mice (Figure 1D).

To ascertain whether the vitamin D_3 increasing effects of β-sitosterol and partly of stigmasterol were caused by changes in lipid levels, cholesterol and triglyceride concentrations in plasma and liver were analyzed. Plasma cholesterol and triglycerides as well as liver cholesterol of the sterol groups did not differ from those of the control group (Table 2). Among the sterol groups, stigmasterol-fed mice had lower concentrations of circulating cholesterol than ergosterol-fed mice and lower circulating levels of triglycerides than ergosterol- and β-sitosterol-fed mice. In contrast, the triglyceride concentration in the liver was significantly lower in the groups fed ergosterol and β-sitosterol and tended to be lower in the stigmasterol group than in the control mice. No correlations were found between the plasma or hepatic concentrations of vitamin D_3 and lipids (triglycerides and cholesterol, respectively), suggesting that the phytosterol-induced change in vitamin D_3 in plasma and tissues was not caused by changes in the lipid levels (Figures 2A–2D).

Because the intestinal uptake of vitamin D depends on micelle and bile acid composition, the fecal bile acid pattern was analyzed. Interestingly, the sterol-fed groups had lower concentrations of secondary bile acids than the control mice (Figure 3A), and there was a significant negative correlation between the secondary bile acids in feces and the vitamin D_3 concentration in the liver (Figure 2F). The most significant differences between the groups were related to muricholic acids, particularly omega-muricholic acid (Table 3 and Figure 3B). More importantly, a negative correlation was found between muricholic acids, in particular omega-muricholic acid, in feces and the vitamin D_3 concentration in the liver (Figures 2G and 2H), which suggests that the vitamin D_3-increasing effect of β-sitosterol and some

Table 2. Body and liver weight, feed intake and lipids in the liver and plasma of mice fed cholesterol-rich diets containing ergosterol, β-sitosterol, stigmasterol or no additional sterols (control)

	Control	Ergosterol	β-Sitosterol	Stigmasterol	P value
Body weight start (g)	14.4±0.6	14.3±0.4	14.5±0.3	14.4±0.3	0.996
Body weight after 4 weeks (g)	22.9±0.5	22.3±0.5	22.3±0.5	22.3±0.5	0.759
Feed intake (g/cage/day)[1]	4.94±0.12	5.05±0.07	5.05±0.08	5.18±0.06	0.276
Liver					
Weight (g)	1.34±0.03^{a}	1.11±0.02^{b}	1.12±0.05^{b}	1.14±0.06^{b}	0.003
Total lipids (mg/g wet weight)	194±8^{a}	165±3^{bc}	156±5^{c}	182±8^{ab}	0.001
Triglycerides (mg/g wet weight)	92.1±5.5^{a}	46.9±5.0^{b}	53.8±5.3^{b}	63.5±9.3^{ab}	<0.001
Cholesterol (mg/g wet weight)	30.3±3.7	26.9±2.5	28.6±2.7	23.8±2.4	0.451
Plasma					
Triglycerides (mmol/l)	0.87±0.06^{ab}	1.05±0.09^{a}	1.08±0.07^{a}	0.74±0.06^{b}	0.006§
Cholesterol (mmol/l)	5.36±0.19^{ab}	5.71±0.23^{a}	5.59±0.18^{ab}	4.79±0.09^{b}	0.041

[1]Two mice per cage. Data are presented as the mean±SEM (n=10, feed intake: n=5). Analyses were performed by one-way ANOVA and Tukey's test or Kruskal-Wallis[§] and Mann-Whitney U test. ^{a,b}Different superscript letters indicate significant differences between the groups (p<0.05).

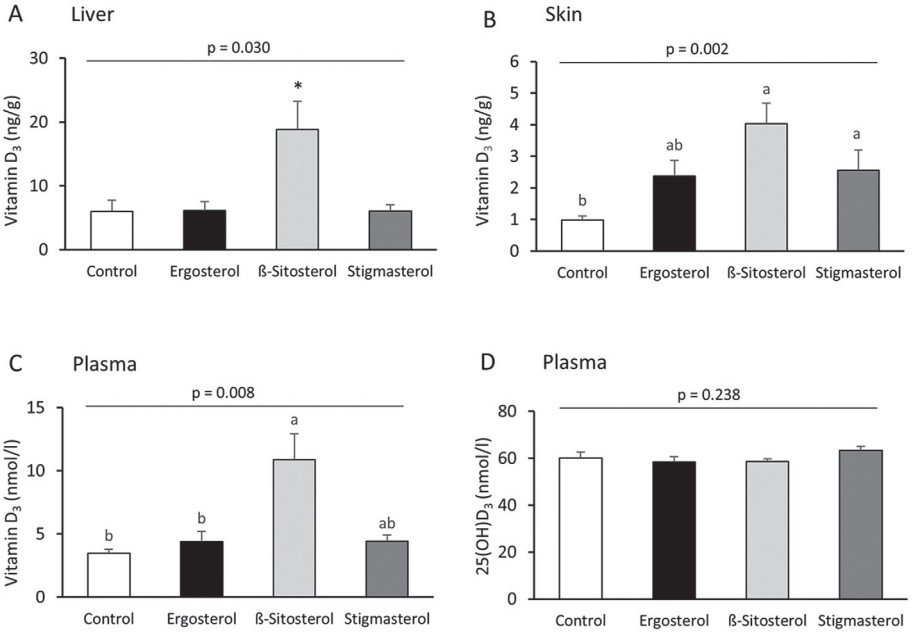

Figure 1. Concentrations of vitamin D_3 in liver (A), skin (B) and plasma (C) and of 25-hydroxy vitamin D_3 (25(OH)D_3) in plasma (D) of mice fed cholesterol-rich diets containing ergosterol, β-sitosterol, stigmasterol or no additional sterol (control) for 4 weeks. Data are presented as the mean±SEM (n=10). Analyses were performed by Kruskal-Wallis and Mann-Whitney U tests. ^{a,b}Different superscript letters indicate significant differences between the groups (p<0.05). *Tends to be different from control (p=0.088).

stigmasterol was caused by differences in the bile acid profile and in turn by improved micellar vitamin D uptake from the gut.

To ascertain whether the changes in vitamin D_3, lipids and bile acids in the groups fed sterols were associated with changes in the transcription levels of relevant intestinal and hepatic genes, the mRNA abundance of intestinal sterol transporters, hepatic enzymes involved in lipid synthesis and fatty acid oxidation, and *Fgf15*, which is a negative regulator of bile acid synthesis [32], was determined. Analyses revealed that the mRNA levels of the intestinal lipid transporters *Npc1l1*, *Cd36*, *Scarb1*, *Abcg5* and *Abcg8* did not differ between the four groups of mice (see Figure E1 and Table E2 in ESM 1). However, the sterol-fed groups had lower mRNA abundance of intestinal *Fgf15* than the control group (control: 1.00±0.33, ergosterol: 0.37±0.12, β-sitosterol: 0.23±0.06, stigmasterol: 0.16±0.03; mean±SEM, p=0.065). Interestingly, in the liver, the mRNA abundance of *Ppara* and its target genes *Acox1* and *Cpt1a* was higher in phytosterol-fed mice than in control mice, while the mRNA expression of genes involved in fatty acid and triglyceride synthesis, such as *Fasn* and *Sc5d,* as well as the transcription factor *Srebf1* remained unaltered (Figure 4).

Discussion

Current analyses showed that the most frequent phytosterols in microalgae are the same, which are commonly found in plants [33]. Phytosterols are generally considered natural substances that impair the bioavailability of

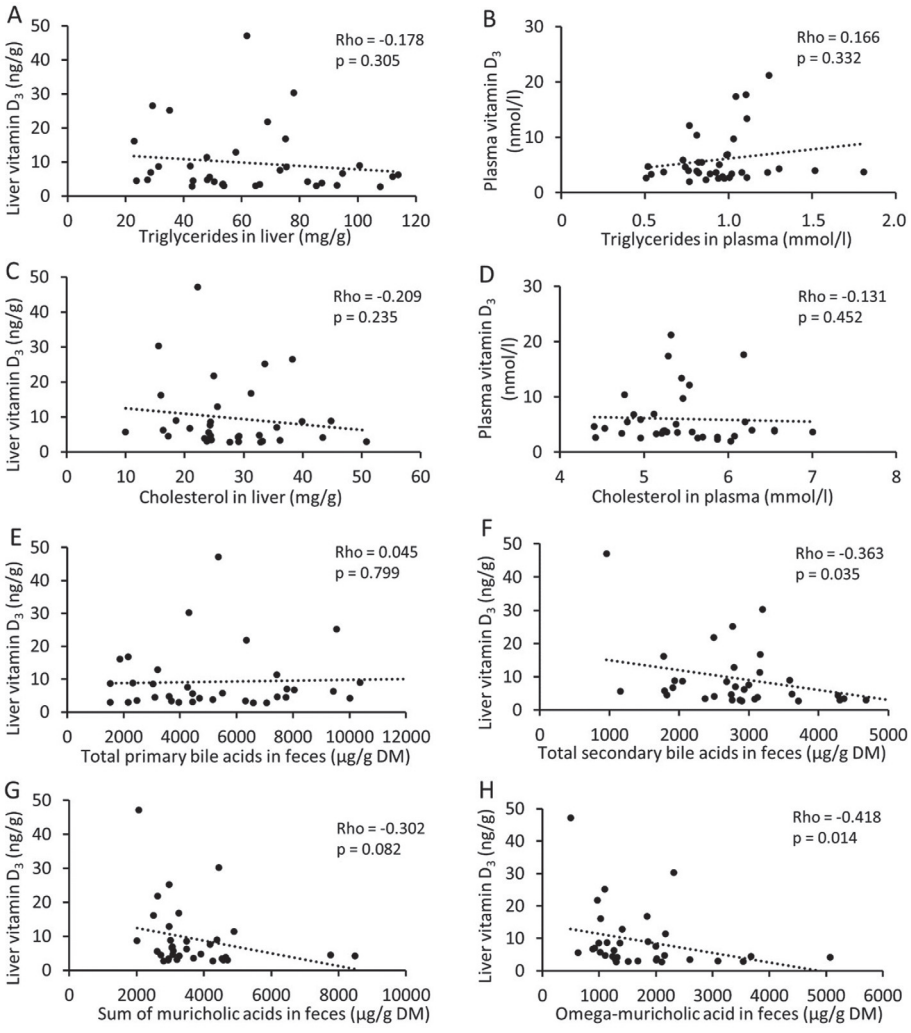

Figure 2. Results of correlation analyses between potential influencing factors on vitamin D_3 concentrations in mice fed cholesterol-rich diets for 4 weeks. Analyses were performed using Spearman correlation. DM: dry matter.

fat-soluble vitamins such as vitamin D. Unexpectedly, the current study showed that β-sitosterol and stigmasterol resulted in higher levels of nonhydroxylated vitamin D_3 in mice, while the 25(OH)D_3 plasma level, which is normally used as a biomarker of vitamin D status, was unaffected. This finding contradicts the hypothesis that phytosterols reduce the absorption of vitamin D, and data from postprandial studies have shown that phytosterols and vitamin D compete for apical uptake in the intestine [18].

Additionally, current data indicate that the observed increases in plasma and tissue vitamin D_3 in phytosterol-fed mice are not caused by changes in triglycerides because lipids and vitamin D_3 levels did not show any correlation. However, it is proposed that intestinal vitamin D uptake is facilitated by a specific bile acid pattern that is entailed by phytosterols. Bile acids are synthesized from cholesterol in the liver and modified by gut bacteria to form secondary bile acids. These bile acids serve as micelle-forming surfactants and determine the efficiency of micelles in carrying hydrophobic nutrients [34]. The important role of bile acids in the intestinal uptake of vitamin D_3 has been well documented [35]. Current analyses of bile acids show that feces of β-sitosterol- and stigmasterol-fed mice were characterized by lower proportions of secondary bile acids, particularly omega-muricholic acid, than control mice. These bile acids were negatively correlated with the vitamin D_3 concentration in the liver. Bile acids largely differ in their hydrophobicity, which in turn influences sterol absorption [36]. It has been demonstrated, e.g., that the administration of hydrophilic muricholic acids resulted in low sterol absorption in mice in comparison with the most hydrophobic bile acids, such as cholic acid and deoxycholic acid [37]. Thus, we speculated that the improved bioavailability of oral vitamin D_3 was attributed to a decrease in the amount of hydrophilic muricholic acids after dietary phytosterol intake. An important regulator of bile acid synthesis is FGF15. This regulator functions as an enterohepatic signal and negative regulator of hepatic bile acid synthesis in response to increased bile acid absorption [38]. The reduced mRNA abundance of intestinal *Fgf15* in

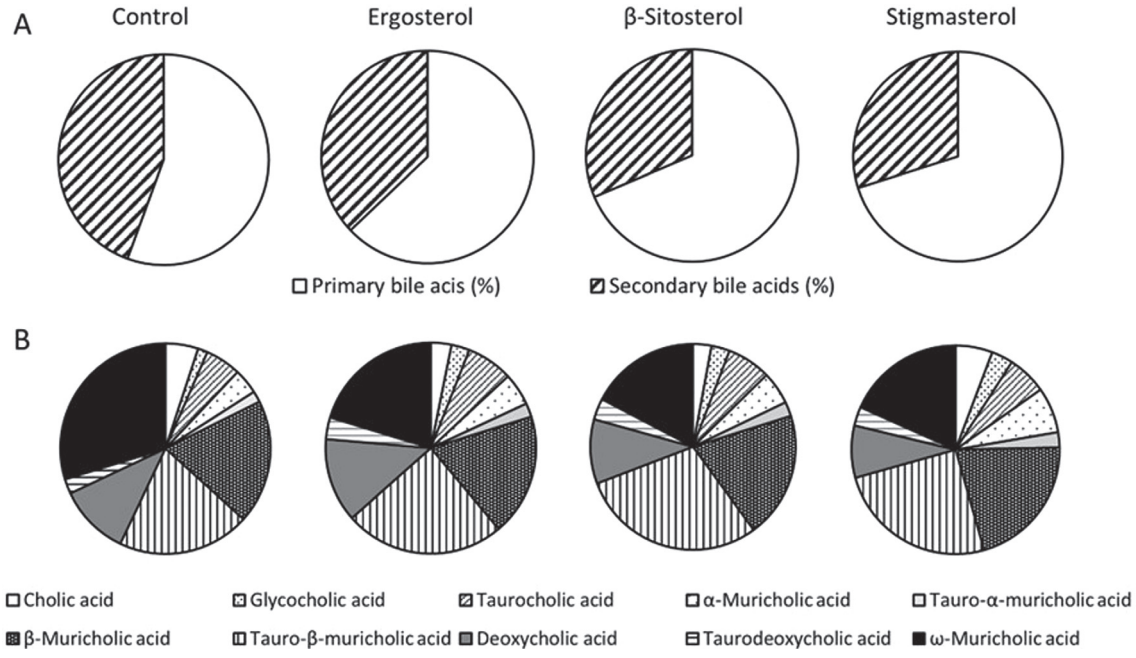

Figure 3. Proportions of primary vs. secondary bile acids (A) and of major bile acids (B) in feces of mice fed cholesterol-rich diets containing ergosterol, β-sitosterol, stigmasterol or no additional sterol (control) for 4 weeks. Data are presented as the mean (n=10).

Table 3. Concentrations of fecal bile acids (μg/g dry matter) of mice fed cholesterol-rich diets containing ergosterol, β-sitosterol, stigmasterol or no additional sterol (control)

	Control	Ergosterol	β-Sitosterol	Stigmasterol	P value
Total bile acids	**9442±1044**	**7450±1035**	**7556±813**	**9186±634**	**0.276**
Total primary bile acids	**5222±1061**	**4661±949**	**5154±714**	**6987±803**	**0.209**
Cholic acid	458±119	227±46	212±18	564±109	0.045§
Glycocholic acid	135±69	200±59	210±55	330±60	0.073§
Taurocholic acid	499±155	510±174	503±128	600±123	0.712§
α-Muricholic acid	354±109^{ab}	357±23^{b}	376±30^{b}	695±85^{a}	0.008§
Tauro-α-muricholic acid	156±57	153±50	166±47	233±41	0.608
β-Muricholic acid	1727±155^{ab}	1337±111^{b}	1440±93^{ab}	2052±245^{a}	0.017
Tauro-β-muricholic acid	1849±671	1762±587	2129±574	2460±384	0.561
Total secondary bile acids	**4172±438^{a}**	**2747±264^{b}**	**2355±273^{b}**	**2971±306^{ab}**	**0.010§**
Deoxycholic acid	991±22	922±103	737±78	795±94	0.061§
Taurodeoxycholic acid	206±82	240±82	244±78	289±58	0.569§
ω-Muricholic acid	2697±418^{a}	1453±169^{ab}	1261±197^{b}	1797±276^{ab}	0.026§
Total tertiary bile acids	**48±9^{ab}**	**42±11^{b}**	**46±7^{b}**	**90±15^{a}**	**0.011§**

Data are presented as the mean±SEM (n=10). Values of individual acids <100 μg/g are not listed. Samples were collected as content of the large intestine. Analyses were performed by one-way ANOVA and Tukey's test or Kruskal-Wallis§ and Mann-Whitney U test. ^{a,b}Different superscript letters indicate significant differences between the groups ($p<0.05$). The total amount of the respective bile acid class is marked in bold.

the phytosterol-fed groups is indicative of altered bile acid metabolism after feeding certain plant or fungal sterols.

Interestingly, despite the higher quantities of vitamin D_3 in the liver, skin and plasma, especially in mice fed β-sitosterol in comparison to the other groups, the plasma concentration of 25(OH)D_3, the classical biomarker of the vitamin D status, remained unchanged. One possible explanation is that plasma 25(OH)D_3 levels do not necessarily correlate with tissue concentrations of vitamin D_3 [39]. An alternative explanation is that vitamin D_3 can not only be hydroxylated to 25(OH)D_3, but also to 20(OH)D_3 through the activity of CYP11A1 [40]. It is therefore possible that the higher amounts of vitamin D_3 in the mice fed β-sitosterol were hydroxylated to a metabolite which was not analyzed in this study. Another interesting observation was that the β-sitosterol treated mice also had substantial amounts of

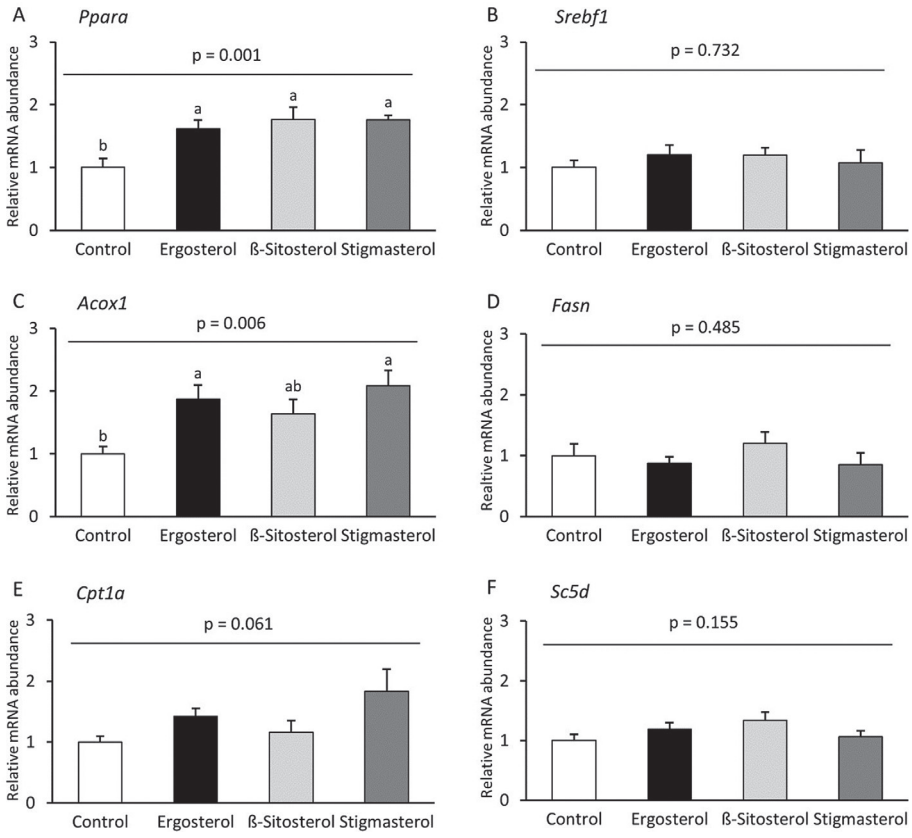

Figure 4. Relative mRNA abundance of genes in the livers of mice fed cholesterol-rich diets containing ergosterol, β-sitosterol, stigmasterol or no additional sterol (control) for 4 weeks. The expression levels of the target genes were normalized to the mean expression levels of *Rplp0* and *Hprt*. Data are presented as the mean±SEM (n=10). Analyses were performed by one-way ANOVA and Tukey's test. [a,b]Different superscript letters indicate significant differences between the groups ($p<0.05$). *Acox1*, acyl-coenzyme A oxidase 1; *Cpt1a*, carnitine palmitoyltransferase 1a; *Fasn*, fatty acid synthase; *Ppara*, peroxisome proliferator activated receptor alpha; *Sc5d,* sterol-C5-desaturase; *Srebf1*, sterol regulatory element binding transcription factor 1.

vitamin D_3 in the skin compared to the other groups. Since all animals were kept in a UVB light-free environment, we assume that skin is capable of storing ingested vitamin D_3.

A second important result was that phytosterols caused a marked reduction in liver triglycerides. Similar findings were observed by Nomaguchi et al. [41], who found that phytosterols from *Aloe vera* had reduced liver triglyceride concentrations in mice fed a high-fat diet. These authors further found that phytosterols can act as ligands for PPARα, which functions as transcription factors and stimulates genes involved in β-oxidation. The current data corroborate these findings because phytosterol-fed mice had a higher mRNA abundance of *Ppara* and corresponding target genes, such as *Acox1* and *Cpt1a*, while genes involved in lipid synthesis remained unchanged. The strong triglyceride-lowering effect of phytosterols may offer great potential to improve steatosis in the liver.

The strengths of this study are (i) the comparison of phytosterols frequently found in microalgae with the fungal ergosterol, (ii) the measurement of non-hydroxylated vitamin D_3 in plasma and selected tissues, besides the analysis of 25(OH)D, to assess the vitamin D status, and (iii) the performance of correlation analyses between vitamin D and lipids and bile acids, respectively.

A limitation of this study is the translatability of data from the mouse study to humans. Although mice are commonly used as a model for studying vitamin D and lipid metabolism, there are certain differences to human metabolism that may limit the extrapolation of the data to humans. One important mouse-human difference is the bile acid profile. Mice in comparison to humans are characterized by an abundant synthesis of 6-hydroxylated bile acids, to which the muricholic acids belong, which have a hydroxy group in the β-configuration at the C-6 position [42]. In contrast, these bile acids are present at very low concentrations in humans. Muricholic acids which were influenced by the phytosterols in the current study have other physicochemical and detergent properties than the classical human bile acids, with 6-hydroxylated bile acids being more water-soluble and relatively poor detergents [42]. This species difference in bile acid composition may limit the ability to translate the findings regarding bile acid-related processes from mice to humans.

In conclusion, microalgae are an important source of phytosterols. However, current data do not indicate that phytosterols may deteriorate the bioavailability of oral vitamin D_3. Furthermore, it must be mentioned that certain microalgae also contain provitamin D_3, which can be converted to vitamin D_3 when exposed to UV-B light [43]. The reduction in liver lipids by activating PPARα and in turn fatty acid oxidation suggest a great health potential of these sterols regarding fatty liver diseases.

Electronic supplementary material

The electronic supplementary material (ESM) is available with the online version of the article at https://doi.org/10.1024/0300-9831/a000766

ESM 1. Schematic illustration of absorption and secretion of cholesterol, plant sterols and vitamin D (Figure E1); Species name, taxonomic classification and cultivation conditions (Table E1); Relative mRNA expression of transporters in duodenal mucosa cells (Table E2).

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Conflict of interest
The authors declare that there are no conflicts of interest.

Author contributions
JK and GIS conceived and designed the mice experiment, CG and SB selected the microalgal species and performed the experimental design for the production of microalgae biomass, BM analyzed the sterols in the microalgae, JK and CB performed the mice experiment and the statistical analyses, JK and MK performed the LC–MS/MS analyses, AN analyzed lipids, JK and GIS wrote the manuscript. All authors critically reviewed the manuscript.

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