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ORIGINAL ARTICLE

Safety aspects and cholesterol-lowering efficacy of low fat dairy products containing plant sterols

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Objective: The aim of this study was to investigate whether a plant sterol mixture would reduce serum cholesterol when added to low fat dairy products in subjects with hypercholesterolaemia, and to examine the effects of the mixture on the serum plant sterol and fat-soluble vitamin levels.
Design: A parallel, double-blind study.
Setting: The study was performed in three different locations in Finland.
Subjects: In total, 164 mildly or moderately hypercholesterolaemic subjects participated in the study.
Methods: The subjects were randomly divided into two groups: a plant sterol group and a control group. The subjects consumed the products for 6 weeks after a 3-week run-in period. The targeted plant sterol intake was 2 g/day in the sterol group.
Results: During the treatment period, there was a 6.5% reduction in serum total cholesterol in the sterol group while no change was observed in the control group ($P<0.0005$). Serum low-density lipoprotein (LDL) cholesterol was reduced by 10.4% in the sterol group and by 0.6% in the control group ($P<0.00005$). There was no change during the trial in serum high-density lipoprotein (HDL) cholesterol or triacylglycerol concentrations. The HDL/LDL cholesterol ratio increased by 16.1% in the sterol group and by 4.3% in the control group ($P=0.0001$). Serum plant sterol levels increased significantly ($P=0.0001$) in the sterol group. None of the fat-soluble vitamin levels decreased significantly when changes in serum total cholesterol were taken into account. The hypocholesterolaemic effect of sterol administration was not influenced by apolipoprotein E phenotype.
Conclusions: Yoghurt, low-fat hard cheese and low-fat fresh cheese enriched with a plant sterol mixture reduced serum cholesterol in hypercholesterolaemic subjects and no adverse effects were noted in the dietary control of hypercholesterolaemia.
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Keywords: cholesterol; LDL cholesterol; hypercholesterolaemia; plant sterols; dairy products; controlled trial

Introduction

Hypercholesterolaemia is a major risk factor for coronary heart disease. Plant sterols reduce serum cholesterol, mainly LDL cholesterol, by inhibiting cholesterol absorption in the small intestine (Heinemann *et al*., 1991; Pouteau *et al*., 2003). Plant sterols proposedly displace cholesterol in the micelles in the intestinal lumen, thus inhibiting the absorption of both dietary and biliary cholesterol (Norman and Wong, 2001). They are structurally similar to cholesterol, differing only in the side chain substitution. Plant stanols are saturated derivatives of sterols. Plant stanols are almost unabsorbable and therefore can be considered as a safe dietary treatment for hypercholesterolaemia. The main

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plant sterols are sitosterol, campesterol and stigmasterol, the major dietary sources being vegetable oils, fats and cereal products (Morton *et al*., 1995). In Western diets, the intake of plant sterols has been estimated to range between 200 and 400 mg/day (Jones *et al*., 1997).

Several clinical trials have demonstrated the potency of plant sterols or stanols as serum cholesterol-reducing substances (Law, 2000; Neil *et al*., 2001; Volpe *et al*., 2001; Mensink *et al*., 2002; Clifton *et al*., 2004; Thomsen *et al*., 2004). Previous studies suggest that at least 0.8–1 g/day of plant sterols should be consumed in order to have a significant reduced serum cholesterol. With a daily consumption of 0.8 g, total cholesterol decreased by 0.26 mmol/l and LDL cholesterol by 0.2 mmol/l (Hendriks *et al*., 1999). Plant sterols have most frequently been added to oily vehicles such as margarines and dressings. There are a few studies showing that a low-fat milk products such as a yoghurt (Volpe *et al*., 2001; Mensink *et al*., 2002; Clifton *et al*., 2004) or low-fat milk (Clifton *et al*., 2004; Thomsen *et al*., 2004) enriched with 1–3 g/day free or esterified plant sterols or stanols has caused a significant decrease in serum total and LDL cholesterol.

The use of plant sterols is considered safe as a part of generally recommended diet. However, the concentration of LDL-adjusted lipophilic vitamins such as carotenoids and tocopherols are lowered by the consumption of plant sterols in some studies but not in all (Berger *et al*., 2004). This is probably due the reduction in absorption, and plant sterol esters seem to lower serum lipophilic vitamin concentration more than free sterols (Richelle *et al*., 2004). The purpose of this study was to investigate the effect of plant sterols mixed with low-fat dairy products (yoghurt, hard cheese and fresh cheese) on important safety parameters such as serum levels of various plant sterols and fat-soluble vitamins, as well as their cholesterol-lowering efficacy in hypercholesterolaemic subjects.

Subjects and methods

Subjects

A total of 207 adult volunteers were screened for a trial in three different centers (Helsinki, Tampere – Ilmomantsi, Oulu). Subjects with serum cholesterol between 5.0 and 8.5 mmol/l and serum triacylglycerols of less than 4.0 mmol/l during the baseline period were included. Patients with severe diseases such as diabetes mellitus, a history of myocardial infarction within the previous 3 months, history of malignancy, psychosis, malabsorption, chronic liver disease or homozygous familiar hypercholesterolaemia were excluded. The use of any hypoglycemic agent, corticosteroids, oral anticoagulants, hormone or immunosuppressant treatment were also exclusion criteria. However, postmenopausal women on stable nonintermittent hormone replacement therapy were accepted. A total of 170 volunteers fulfilled the inclusion criteria and were randomly assigned either to a plant sterol-containing low-fat yoghurt group (Helsinki), a low-fat hard cheese group (Tampere – Ilomantsi) or a low fat fresh cheese diet group (Oulu), or the control group (Helsinki, Tampere –Ilmomantsi, and Oulu). During the study, the patients were not allowed to use any lipid-lowering drugs or a lipid-lowering dietary regime. Of these subjects, 164 (82 in the active group and 82 in the placebo group) completed the study. Five of the randomised subjects voluntarily withdrew from the study and one died of cardiovascular disease during the trial. None of the subjects (128 women and 36 men) had a recent history of cholesterol-lowering drug treatment. The subjects were requested to maintain their normal diet, weight and physical activity during the study. The baseline characteristics of the subjects are shown in Table 1.

The study protocol was approved by the Ethics Committee of the Department of Medicine, Helsinki University Central Hospital. All the subjects received both written and oral information regarding the trial and gave their written consent.

Study design

The trial was carried out using a parallel-group double-blind design. The screening period before the random allocation of the subjects into one or other of the two study groups lasted

Table 1 Baseline characteristics of the subjects[a]

Variables	*Sterol group*[b] *(n = 82)* *Mean ± s.d.*	*Control group*[c] *(n = 82)* *Mean ± s.d.*
Age (years)	57.6 ± 9.1	57.0 ± 8.4
Women/men	65/17	64/18
Weight (kg)		
Men	83.4 ± 11.5	83.9 ± 12.5
Women	70.2 ± 10.6	69.3 ± 10.7
Body mass index (kg/m^2)	27.1 ± 3.9	26.8 ± 4.2
Waist/hip ratio		
Men	0.96 ± 0.05	0.93 ± 0.06
Women	0.83 ± 0.05	0.84 ± 0.05
Serum lipids (mmol/l)		
Total cholesterol	6.4 ± 0.7	6.4 ± 0.6
LDL cholesterol	4.1 ± 0.6	4.0 ± 0.7
HDL cholesterol	1.7 ± 0.4	1.7 ± 0.4
HDL/LDL cholesterol ratio	0.4 ± 0.1	0.4 ± 0.2
Triacylglycerols[d]	1.3 ± 0.6	1.4 ± 0.6
Blood pressure (mmHg)		
Systolic	132 ± 14	126 ± 14
Diastolic	82 ± 7	81 ± 8

[a]Weight, BMI, lipids and blood pressure measurements are from visit three (randomisation). There are no statistically significant differences between groups.
[b]$n = 82$.
[c]$n = 82$.
[d]$n = 78$ in the sterol group.

3 weeks. During the last week of the 3-week-long screening period all the subjects received the control food items. During the treatment period the subjects received either sterol-containing study food items or control food items. Otherwise, no restrictions were imposed on their diets.

At each of the three visits during the screening period (weeks −3, −1 and 0) the serum lipid concentrations were determined. After randomisation, the double-blind dietary trial period lasted about 6 weeks (34–49 days). During the treatment period, the subjects had two study visits, at weeks 3 and 6. Fasting blood samples were taken and body weight was recorded at each visit. In addition, data concerning medical history, use of drugs for other diseases, smoking habits, and physical activity were obtained during the clinical visits by means of a questionnaire. During the dietary trial period any adverse effects were recorded at each visit. After completing the trial period, the subjects attended another follow-up visit 1–7 weeks (7–53 days) later once they had returned to their usual diet. At the follow-up visit the subjects received dietary advice, if they wished, from the study nutritionist.

Study food items

The dietary products enriched with plant sterols (and the matched placebo products) were (i) flavoured low-fat yoghurt, (ii) low-fat hard cheese or (iii) low-fat fresh cheese. The study subjects received either 150 g of yoghurt per day, or 50 g of hard cheese or fresh cheese per day. In the hard cheese and fresh cheese groups the daily amount of the mixture of plant sterols and stanols was about 2 g in the active diet group while it was somewhat less in the yoghurt group. The compositions of the study items are presented in Table 2. In the control group, the food items were identical to the study products but without the added plant sterol mixture. The mixture of plant sterols was extracted from Pine trees, mainly *Pinus maritime L*. The plant sterol mixture was added to the other ingredients of the low-fat milk products using a mechanical mixing method. The plant sterols were free sterols and stanols in crystalline form. The plant sterol mixture consisted of 75% β-sitosterol, 10% β-sitostanol, 10% campesterol, 2% campestanol, and 2% other sterols.

The products were stored at the study clinic and were dispensed to the subjects at 1–3-week intervals. The subjects recorded their use of the study products in a consumption diary, which was checked at each study visit. Any deviations from the planned regime and the reasons for this were recorded. According to the consumption diaries, the mean consumption of the study food items was 149 g/day in the yoghurt group (range 136–150 g/day), 49 g/day in the hard cheese group (47–50 g/day) and 49 g/day in the fresh cheese group (40–50 g/day).

Diet

Three times during the study all the subjects filled in a 3-day food record, which included two weekdays and one weekend day. The first such period was during the first baseline weeks (home diet), the second during the last week of the 3-week-long run-in period when a placebo was ingested, and the second during the randomised diet period. Portion sizes were

Table 2 Composition of plant sterol-containing yoghurt, low-fat plant sterol-containing hard cheese and fresh cheese and control (standard) food items

Nutrients	*Yoghurt*		*Hard cheese*		*Fresh cheese*	
	Sterol (150 g)	*Control (150 g)*	*Sterol (50 g)*	*Control (50 g)*	*Sterol (50 g)*	*Control (50 g)*
Energy (kJ)	536	540	475	475	375	375
Protein (g)	5.25	5.25	15.5	15.5	3.25	3.25
Carbohydrates (g)	19.5	19.5	1.4	1.4	3.95	4
Total fat (g)	3.0	3.0	5	5	7	7
Fatty acids						
Saturated (g)	1.95	1.95	3	3	4.0	4.0
Monounsaturated (g)	0.6	0.6	1	1	1.35	1.35
Polyunsaturated (g)	0	0	0.1	0.1	0.15	0.15
Cholesterol	7.8	7.8	13	13	18	18
Plant sterols (g)	1.65	0	2	0	2	0
β-Sitosterol (g)[a]	1.24		1.5		1.5	
β-Sitostanol (g)[a]	0.16		0.2		0.2	
Campesterol (g)[a]	0.16		0.2		0.2	
Campestanol (g)[a]	0.03		0.04		0.04	
Other plant sterols (g)[a]	0.03		0.04		0.04	
Milk fat (g)	0.75	3.0	4.5	7.5	4–5	6–7
Vegetable fat (g)	0.6	0	0.6		0.6	

Values are per daily dose of the product.
[a]Plant sterol amounts are calculated from the manufacturer's information of plant sterol powder (75% β-sitosterol, 10% β-sitostanol, 10% campesterol, 2% campestanol, 2% others).

estimated using a validated portion size picture booklet (Pietinen *et al*., 1988). The food records were checked at the clinical visits by a nutritionist. Nutrient intakes were calculated using the programme developed at the National Public Health Institute, Helsinki, Finland (Ovaskainen *et al*., 1996).

Serum lipids and apolipoprotein E phenotype

Blood samples were obtained after the subjects had been fasting for at least 12 h. Serum lipid levels were determined at each study visit. High-density lipoprotein (HDL) cholesterol and triacylglycerol levels were also determined, except at the second study visit. The assays were performed at the Laboratory of Analytical Biochemistry of the National Public Health Institute. Total serum cholesterol was analysed by the enzymatic CHOD-PAP colorimetric test (Thermo Clinical Labsystems, Vantaa, Finland), HDL cholesterol by the direct method (Thermo Clinical Labsystems, Vantaa, Finland) (Sugiuchi *et al*., 1995), and triacylglycerols by the enzymatic GPO-PAP colorimetric test (Thermo Clinical Labsystems, Vantaa, Finland), using the Optima Clinical Chemistry Analyszer (Thermo Clinical Labsystems, Vantaa, Finland). The low-density lipoprotein (LDL) cholesterol concentration was calculated with Friedewald's formula (Friedewald *et al*., 1972). Apolipoprotein E (Apo E) phenotyping was performed by isoelectric focusing (Havekes *et al*., 1987).

Serum plant sterols

The phytosterols in the serum were determined using an application of the GC method published by Phillips *et al*. (1999), which was modified by adding a further sample extract purification step. The extract was introduced to a silica solid phase extraction cartridge (Varian Bond Elut, 500 mg) that had been activated with hexane. The cartridge was washed with hexane and with hexane:diethyl ether (90:10, v/v). The sterols were eluted with hexane:diethyl ether (50:50, v/v).

For the daily quality control, an in-house serum reference sample was analysed in each sample assay. The plant sterol contents of the in-house serum reference sample remained stable during the study. Plant sterol and lathosterol contents ($n=55$) were as follows: 5.6 ± 0.6 mg/l for campesterol, 2.1 ± 0.2 mg/l for sitosterol, 0.2 ± 0.1 mg/l for stigmasterol, 0.5 ± 0.04 for Δ5-avenasterol and 2.3 ± 0.2 mg/l for lathosterol.

Vitamins

The plasma 25(OH) vitamin D concentration was measured by radioimmunoassay (Incstar Corporation, Stillwater, MN, USA). The intra- and interassay coefficients of variation were 10.1 and 14.9%, respectively. The reference range for 25(OH)D was 25–120 nmol/l.

The α- and γ-tocopherol, β-carotene, all-*trans*-retinol and phylloquinone (vitamin K_1) concentrations in plasma were determined using a method composed mainly of the HPLC applications published by Chuang *et al*. (1994) and Koivu-Tikkanen *et al*. (2000). All the vitamins were determined in the same extract (Koivu-Tikkanen *et al*., 2005). α- And γ-tocopherol, β-carotene and the all-*trans*-retinol were determined simultaneously in the same HPLC run (Koivu-Tikkanen *et al*., 2005), whereas phylloquinone was determined after further purification of the extract using a separate HPLC system (Koivu-Tikkanen *et al*., 2000).

For quality control of the method, a certified reference material (NIST SRM 986 c) was analysed. The results of the all-*trans*-retinol, β-carotene, α- and γ-tocopherol analyses were within the certified ranges. For daily quality control an in-house reference plasma was analysed in every second sample assay. The day-to-day repeatabilities (CV %, $n=38$) were 7.9% for all-*trans*-retinol, 9.6% for β-carotene, 2.4% for α- tocopherol, 4.8% for γ-tocopherol and 9.6% for phylloquinone.

Oxidation of LDL in vitro

LDL was isolated from EDTA plasma for the oxidation experiments, by first separating VLDL by ultra centrifugation in a Beckman Ti 50.4 rotor (269 000 g, 3.5 h, $+15^{\circ}$C). Following the removal of the VLDL fraction by slicing the centrifuge tubes, LDL was separated, using the same rotor (155 000 g, 18 h, $+15^{\circ}$C), and isolated again by slicing.

EDTA was removed by gel-filtration on Sephadex G-25 in a phosphate-buffered saline solution (PBS). EDTA, 60 mM, was added to a final concentration of 1.2 mM. Copper mediated oxidation was begun: gel-filtered LDL (100 μg protein/ml) in PBS (pH 7.4) was supplemented with $CuSO_4$ to a final concentration of 10 μM. The diene absorption at 234 nm was recorded at $+20.5^{\circ}$C with a Shimadzu UV-1202 spectrophotometer (Shimadzu Corp., Kyoto, Japan) equipped with a cell temperature CPS controller (Shimadzu Corp., Tokyo, Japan). When diene formation reached its maximum, oxidation was stopped by adding EDTA as above. Oxidation experiments were carried out twice at 48 h intervals, and mean values were used for calculations. The protein content of LDL was analysed by the method of Lowry *et al*. (1951). The lag phase or inhibition period is the time when the rate of oxidation is slow.

Other observations and measurements

Body weight (to 0.1 kg) in light indoor clothing without shoes was recorded at every visit. Heart rate (60 s) and blood pressure (2 mmHg precision) were recorded twice with a mercury sphygmomanometer on the subjects who were in a seated position. Height (0.5 cm precision) was measured at the beginning of the study. Waist and hip circumferences were measured twice, and the subjects were also interviewed regarding their health habits and smoking and drug use, first at the beginning and again at the end of the study. Physical activity and changes in drug use and health status were

recorded at every visit. At the end of the study the subjects were asked to report, anonymously, any possible adverse effects.

Statistical analyses

Normality of distribution of the dependent variables was confirmed using the Shapiro–Wilks test. Differences in changes in serum lipids before (randomisation visit) and after the trial period were tested between the sterol group and the control group (Student's *t*-test) or with the Wilcoxon test for non-normal variables. The test for homogeneity showed that there were no statistically significant differences in the responses on lipid levels or on other parameters between the three different product groups (yoghurt, hard cheese and fresh cheese) within the sterol group; therefore, it was possible to pool the data from these three groups. *P*-values were computed for the differences between the randomisation groups. The χ^2 test was used to test differences between the randomisation groups for categorised variables (e.g. smoking habits, adverse effects). The results are expressed in the tables as means$\pm$s.d. and in the figures as means$\pm$s.e.m. Statistical analyses were carried out with SAS (Statistical Analysis System) version 6.12 software (SAS Institute Inc., Cary, NC, USA).

Results

There were no significant differences in the baseline characteristics between the study groups (Table 1). Body weight changed only slightly during the trial (-0.3 ± 1.0 and -0.2 ± 1.1 kg in the sterol and control groups, respectively). However, there was a significant difference between the groups in the change in systolic blood pressure (-4 ± 5 and 0 ± 10 mmHg in the sterol and control groups, respectively). Smoking habits did not change, nor did physical activity change significantly between the groups during the trial. There were no differences in adverse effects between the groups during the study.

Nutrient intake

There were no statistically significant differences in the nutrient intake between the randomised groups at baseline (home diet) or during the experimental period (data not shown). There were only a few significant differences in the changes of nutrient intake between the run-in and the experimental periods between the randomised groups (Table 3). After correction for multiple comparisons, none of the differences remained statistically significant, except for the change in energy intake from alcohol, which increased more in the sterol group than in the control group ($P=0.0046$).

Serum lipids

The serum total and LDL cholesterol concentrations during the run-in, treatment and follow-up periods are shown in Figure 1. Serum total cholesterol decreased in the sterol group by 4% in the yoghurt group, by 8% in the hard cheese group, by 7% in the fresh cheese group and by 7% in all the groups combined. LDL cholesterol decreased by 9% in the yoghurt group, by 11% in the hard cheese and the fresh cheese groups and by 10% in all the groups combined (Table 4). There were no significant differences in changes in HDL cholesterol and triacylglycerols between the sterol and control groups (data not shown). Nor there were significant differences in the total or LDL cholesterol responses between the three randomised groups.

Serum plant sterols and lathosterol

The total concentration of serum plant sterols increased in the sterol group ($P=0.0001$), but not in the control group

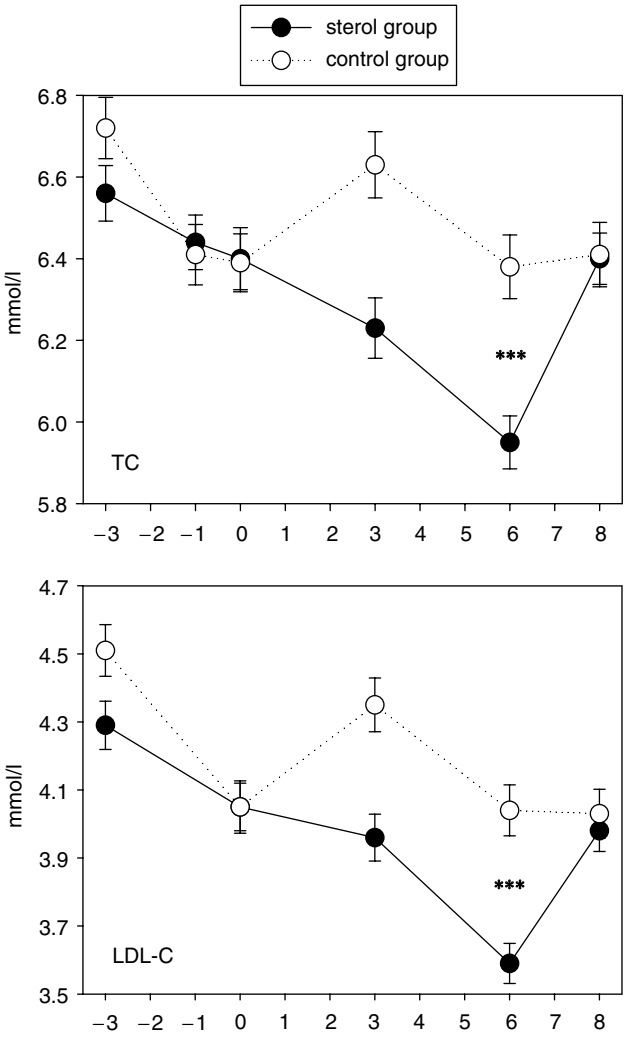

Figure 1 Serum lipid concentrations of the sterol group ($n=82$) and the control group ($n=82$) during the trial. TC = total cholesterol, LDL-C = low-density lipoprotein cholesterol. ***P-value <0.001. The values are expressed as mean$\pm$s.e.m.

Table 3 Nutrient intake during trial (calculated from 3-day recalls) (mean±s.d.)

Nutrients	*Group*[a]	*Run-in period*	*Experiment period*	*Change between run-in and treatment periods*	*Test for the change between groups* P-*value*
Energy (MJ/day)	Sterol[b]	7.7±2.0	7.6±2.1	−0.05±1.7	
	Control	7.6±2.3	7.5±2.2	−0.07±1.5	0.9305[c]
Fat (% of energy)	Sterol[b]	33.8±5.6	33.7±5.8	−0.08±4.7	
	Control	33.3±4.8	34.1±4.8	0.8±5.9	0.3088[c]
Fatty acids (% of energy)					
Saturated	Sterol[b]	15.6±3.3	15.9±3.7	0.4±3.2	
	Control	15.2±3.4	16.1±3.1	0.9±3.0	0.2908[c]
Monoun-saturated	Sterol[b]	12.3±2.4	12.3±2.6	0.04±2.3	
	Control	12.1±2.3	12.4±2.0	0.2±2.9	0.6420[c]
Polyunsaturated	Sterol[b]	5.2±1.7	4.8±1.2	−0.5±1.5	
	Control	5.2±1.7	4.9±1.3	−0.3±2.1	0.3720[c]
Protein (% of energy)	Sterol[b]	17.8±3.3	17.3±3.4	−0.5±3.5	
	Control	18.6±3.3	17.9±2.8	−0.7±3.4	0.6465[c]
Carbohydrate (% of energy)	Sterol[b]	46.9±6.4	46.2±6.0	−0.9±5.4	
	Control	45.9±5.9	45.3±6.6	−0.5±6.5	0.7232[c]
Alcohol (% of energy)	Sterol[b]	1.4±3.1	2.8±4.7	1.4±3.9	
	Control	2.2±4.2	2.6±4.7	0.5±4.7	0.0046[d]
Cholesterol (mg/MJ)	Sterol[b]	36.9±12.1	36.9±12.9	0.2±14.4	
	Control	36.1±12.3	37.1±13.2	1.0±14.1	0.7152[c]
Fiber (g/MJ)	Sterol[b]	2.5±0.7	2.3±0.8	−0.2±0.7	
	Control	2.4±0.9	2.2±0.7	−0.2±0.8	0.6386[d]
Vitamin A (RE, μg)	Sterol[b]	947±894	793±905	−154±1223	
	Control	998±1717	791±605	−206±1741	0.0624[d]
Retinol (μg)	Sterol[b]	506±853	431±823	−73±1173	
	Control	577±1652	430±506	−147±1661	0.0124[d]
Carotenoids (μg)	Sterol[b]	5357±3398	5488±3362	105±3277	
	Control	5083±3464	5859±3013	776±3729	0.2244[c]
β-Carotene (μg)	Sterol[b]	2224±1990	1743±1488	−493±2148	
	Control	2203±2187	1841±1489	−362±2119	0.3675[d]
Vitamin D (μg)	Sterol[b]	5.4±5.0	5.5±5.2	0.2±6.5	
	Control	6.4±7.1	5.6±4.9	−0.8±7.6	0.4683[d]
Vitamin E (TE, mg)	Sterol[b]	9.4±3.8	8.0±2.6	−1.4±3.4	
	Control	8.6±3.5	8.0±2.7	−0.5±3.7	0.4912[c]
Vitamin K_1 (μg)	Sterol[b]	79±35	62±24	−17.5±31.0	
	Control	68±34	64±33	−3.2±36.7	0.0229[d]

[a]In the sterol group $n=82$ and in the control group $n=82$.
[b]In the sterol group, one did not return diary record at the treatment period ($n=81$).
[c]*t*-Test between groups.
[d]Wilcoxon test for non-normal variables between groups.

(Table 5). Sitosterol concentration increased significantly in the sterol group but not in the control group ($P=0.0001$). The avenasterol concentration decreased in the sterol group only ($P=0.0001$) and campesterol also showed a decreasing trend ($P=0.0669$). The changes in serum lathosterol did not differ significantly in the sterol and control groups.

Plasma fat-soluble vitamins

There were no significant differences between the groups in plasma γ-tocopherol, retinol, vitamin K_1 or 25(OH) vitamin D concentrations during the trial (Table 6). Plasma α-tocopherol decreased more in the sterol diet group than in the control group. β-Carotene also decreased in the sterol

Table 4 Changes (%) in serum lipids between sterol and control group in different food item groups[a] (mean ± s.d.)

	Yoghurt	*Hard cheese*	*Fresh cheese*	*All groups together*
Total cholesterol				
Sterol	−4.0 ± 8.9	−8.3 ± 10.1	−6.6 ± 7.0	−6.5 ± 9.0
Control	−0.9 ± 8.1	−1.4 ± 7.7	2.3 ± 8.2	−0.0 ± 8.1
P-value[b]	0.3417[c]	0.0038	0.0001	<0.00005
LDL cholesterol				
Sterol	−8.7 ± 11.7	−11.2 ± 13.6	−11.2 ± 8.3	−10.4 ± 11.6
Control	−1.0 ± 11.5	0.03 ± 11.3	2.6 ± 13.4	0.6 ± 12.0
P-value[b]	0.0313[d]	0.0009	0.0001[c]	<0.00005

[a]Means ± s.d., in sterol group, $n = 25$ (yoghurt), $n = 33$(hard cheese), $n = 24$ (fresh cheese), $n = 82$ (all together), in control group, $n = 25$ (yoghurt), $n = 29$(hard cheese), $n = 28$ (fresh cheese), $n = 82$ (all together).
[b]*t*-Test for normal variables between randomisation groups.
[c]Wilcoxon test for non-normal variables between randomisation groups.
[d]Three from yoghurt sterol group and one from cheese sterol group are excluded from analysis.

group while it increased in the control group. However, there were no significant differences between the groups when α-tocopherol and β-carotene were related to serum total cholesterol (Table 6).

Apo E phenotype

There were no differences in the total and LDL cholesterol reductions between the treatment groups when the subjects were stratified according to Apo E phenotype (Apo E 32 + 33 and Apo E 43 + 44): in both strata, total and LDL cholesterol decreased significantly more in the sterol group than in the control group (data not shown). Nor did changes in serum plant sterol concentrations differ between Apo E phenotypes (data not shown). In both Apo E phenotypes the serum sitosterol increased more and serum avenasterol and stigmasterol decreased more in the sterol group than in the control group (data not shown).

LDL oxidation

No statistically significant differences between the sterol and the control groups were seen in the rate of LDL oxidation, maximal oxidation, lag phase or diene formation. At the end of the treatment period the lag phase was 141.7 ± 30.6 min (change −22.22 min) in the sterol group and 149 ± 23.6 min (change −10.42 min) in the control group ($P = 0.3515$ between the groups).

Discussion

This double-blind, randomised study showed that low-fat yoghurt, low-fat hard cheese and low-fat fresh cheese enriched with a plant sterol mixture reduced serum total and LDL cholesterol levels in mildly or moderately hypercholesterolaemic subjects during the 6-week trial. In previous studies, the reduction in serum LDL cholesterol was observed after 1–4 weeks of plant sterol consumption, with a return to its initial level within 2–3 weeks after the end of the consumption (Pelletier *et al*., 1995; Weststrate and Meijer, 1998; Mensink *et al*., 2002).

In this study, the consumption of low fat dairy products (yoghurt, hard cheese and fresh cheese) enriched with a 1.7 g sitosterol mixture/day (yoghurt) or a 2 g sitosterol mixture/day (hard cheese and fresh cheese) resulted in a significant decrease in serum total cholesterol and LDL cholesterol. There were no statistically significant differences between the three food item groups: LDL cholesterol was reduced by approximately 10% whereas HDL cholesterol and triacylglycerols did not change during the trial. As the dietary treatment periods varied between 34 and 49 days and the daily sterol intake was 1.7 g in the yoghurt group and 2 g in both cheese groups, the food item groups cannot be compared exactly. However, the results indicate that the cholesterol-lowering efficacy was comparable with previous studies.

In earlier studies, plant sterols (1–5 g/day) have reduced total cholesterol and LDL cholesterol by 5–13 and by 7–16% (Law 2000; Neil *et al*., 2001; Tikkanen *et al*., 2001). There are only a few studies showing that low-fat milk products such as yoghurt-based drinks (Volpe *et al*., 2001; Mensink *et al*., 2002; Clifton *et al*., 2004) or milk (Clifton *et al*., 2004; Thomsen *et al*., 2004) enriched with 1–3 g/day free or esterified plant sterols or stanols cause a significant decrease in serum total and LDL cholesterol.

The cholesterol-lowering effect of plant sterols has usually been attributed to β-sitosterol. In the present study, the mixture of plant sterols consisted mainly of β-sitosterol but it also contained a number of other sterols and stanols: β-sitostanol, campesterol, campestanol and small quantities of other sterols.

Previous studies have shown that the effects of plant sterols are dose dependent up to a dose of 2 g of plant sterols per day (Hendriks *et al*., 1999; Thomsen *et al*., 2004) at higher plant sterol doses only a slight additional decrease in LDL cholesterol levels is observed (Hendriks *et al*., 1999). It has also been suggested that total and LDL cholesterol concentrations and the characteristics of the subjects can influence the efficacy of plant sterols (Gylling *et al*., 1997).

Plant sterols have not been found to cause obvious adverse effects or biochemical anomalies in humans (Jones *et al*., 1997). In our study, the plant sterol powder added to the milk products mainly consisted of β-sitosterol and it was probable that serum concentrations of plant sterols to some degree increased since the dosage was higher than in many earlier studies. It seems that sitosterol displaces other plant sterols, at least avenasterol. Serum plant sterol levels remained, however, within the normal ranges. Serum plant stanol levels were not determined because the plant stanol concentration was under the quantitation limit (0.1 mg/l). In

Table 5 Serum plant sterols and lathosterol levels during the trial $n=82$ in the control group and in the sterol groups in each measurement

	Baseline (0 week) Mean±s.d.	*Treatment period (6 weeks)* Mean±s.d.	*Change* Mean±s.d.	*Test for change between groups* P-value
Campesterol (mg/l)				
Sterol	7.5±3.0	6.9±2.5	−0.6±1.3	
Control	6.8±3.2	6.7±3.1	−0.2±1.2	0.0669[a]
Sitosterol (mg/l)				
Sterol	2.9±1.2	4.4±1.7	1.6±1.1	
Control	2.6±1.3	2.6±1.2	−0.1±0.5	0.0001[a]
Stigmasterol (mg/l)				
Sterol	0.3±0.2	0.3±0.2	−0.0±0.1	
Control	0.3±0.2	0.3±0.2	−0.0±0.1	0.2765[a]
Avenasterol (mg/l)				
Sterol	0.9±0.3	0.7±0.2	−0.1±0.2	
Control	0.8±0.3	0.8±0.3	−0.0±0.2	<0.00005[b]
Sum of plant sterols (mg/l)				
Sterol	11.5±4.4	12.3±4.2	0.8±2.1	
Control	10.6±4.7	10.3±4.6	−0.3±1.7	0.0001[a]
Lathosterol (mg/l)				
Sterol	3.0±1.2	3.1±1.4	0.1±0.8	
Control	3.4±1.5	3.4±1.5	0.0±0.7	0.6427[a]

[a]Wilcoxon test between groups.
[b]*t*-Test between groups.

Table 6 Plasma fat-soluble vitamins and ratios to serum total cholesterol during the trial $n=81$ in the control group and $n=82$ in the sterol groups

	Baseline (0 weeks) Mean±s.d.	*Treatment period (6 weeks)* Mean±s.d.	*Change* Mean±s.d.	*Test for change between groups* P-value
γ-Tocopherol (μg/l)				
Sterol	852±337	815±328	−37±244	
Control	798±355	775±332	−23±246	0.7387[a]
γ-Tocopherol/TC[b] (μg/mmol)				
Sterol	135±57	137±53	2.3±42.1	
Control	127±59	122±50	−4.8±43.2	0.2019[a]
α-Tocopherol (mg/l)				
Sterol	14.2±2.9	13.1±2.9	−1.1±2.4	
Control	14.4±2.9	14.4±3.2	0.0±1.7	0.0004[a]
α-Tocopherol/TC[b] (mg/mmol)				
Sterol	2.2±0.4	2.2±0.4	−0.01±0.3	
Control	2.3±0.4	2.3±0.4	0.003±0.2	0.6104[a]
β-Carotene (μg/l)				
Sterol	316±172	307±152	−9±74	
Control	352±228	385±205	33±104	0.0002[a]
β-Carotene/TC[b] (μg/mmol)				
Sterol	50±27	52±28	2.6±13.0	
Control	55±35	60±31	5.1±16.2	0.0823[a]
Retinol (μg/l)				
Sterol	432±89	420±79	−11±57	
Control	428±84	429±97	0.9±57	0.1809[c]
Vitamin K_1 (μg/l)				
Sterol	0.49±0.45	0.41±0.36	−0.08±0.37	
Control	0.37±0.27	0.36±0.30	−0.02±0.34	0.7089[a]
25 (OH) vitamin D (μg/l)				
Sterol	13.7±6.3	17.5±6.2	3.8±5.0	
Control	13.4±4.7	16.7±5.2	3.3±4.1	0.2933[a]

[a]Wilcoxon test between groups.
[b]TC = Total cholesterol.
[c]*t*-Test between groups.

this study, plant sterols were well tolerated and the subjects did not report any adverse effects. Nor was any significant effect on LDL oxidation observed.

No significant effects on serum concentrations of 25(OH) vitamin D and retinol were observed which is in line with other studies (Law, 2000). The intake of vitamin D-containing dairy products apparently contributed to the increase in serum vitamin D concentrations. Plasma α-tocopherol and β-carotene concentrations decreased significantly in the sterol diet group compared to the control group. These fat-soluble vitamins are transported in serum lipoproteins, mainly in LDL and VLDL. The differences in the changes between the sterol and the control groups were no longer significant when the α-tocopherol and β-carotene values were adjusted for the reduced serum total cholesterol concentrations. This accords with some earlier studies with free plant sterols (Volpe *et al*., 2001; Colgan *et al*., 2004; Thomsen *et al*., 2004) or with plant sterol esters (Nestel *et al*., 2001; Raeini-Sarjaz *et al*., 2002). However, in other studies with free plant sterols (Weststrate and Meijer, 1998; Hallikainen *et al*., 1999) or with plant sterol esters (Gylling *et al*., 1999; Hendriks *et al*., 1999; Mensink *et al*., 2002), there has been a significant (8–30%) reduction in β-carotene or α-tocopherol concentrations after lipid adjustment for reduced total or LDL cholesterol. It has been suggested that esterified plant sterols have a greater effect than free plant sterols on the reduction of fat-soluble vitamin absorption because the plant sterol esters partition into the oil phase of the intestine, whereas free sterols partition more frequently into the mixed micellar phase (Nissinen *et al*., 2002). Details of the regulation of the lipophilic nutrient absorption are largely unknown and this needs further investigation.

In conclusion, the present study showed that yoghurt, hard cheese and fresh cheese enriched with plant sterols resulted in total and LDL cholesterol-lowering effects in hypercholesterolaemic subjects. The hypocholesterolaemic effect and the effect on plasma sterol concentrate did not depend on Apo E phenotype. The subjects did not report any adverse effects. The results indicate that a daily intake of 2 g of plant sterol mixture did not influence cholesterol-adjusted vitamin levels and only increased the total plasma sterol level by 7%. Although these important safety parameters remained at acceptable levels, the data do not allow conclusions as to the long-term safety of plant sterol intake.

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References

Berger A, Jones PJH, Abumweis SS (2004). Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lip Health Dis* **3**, 1–19.

Chuang CH, Trosclair G, Lopez-S A (1994). Adaptation of a carotenoid procedure to analyze carotenoids, retinol and alpha-tocopherol simultaneously. *J Liquid Chromatogr* **17**, 3613–3622.

Clifton PM, Noakes M, Sullivan D, Erichsen N, Ross D, Annison G *et al*. (2004). Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal. *Eur J Clin Nutr* **58**, 503–509.

Colgan HA, Floyd S, Noone EJ, Gibley MJ, Roche HM (2004). Increased intake of fruit and vegetables and a low-fat diet, with and without low-fat plant sterol-enriched spread consumption: effects on plasma lipoprotein and carotenoid metabolism. *J Hum Nutr Dietet* **17**, 561–569.

Friedewald WT, Levy RI, Fredrickson DS (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **18**, 499–502.

Gylling HK, Puska P, Vartiainen E, Miettinen TA (1999). Retinol, vitamin D, carotenes and alpha tocopherol in serum of a moderately hypercholesterolaemic population consuming sitostanol margarine. *Atherscl erosis* **145**, 279–285.

Gylling H, Radhakrishnan R, Miettinen TA (1997). Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary sitostanol ester margarine: women and dietary sitostanol. *Circulation* **96**, 4226–4231.

Havekes LM, de Knijff P, Beisiegel U, Havinga J, Smit M, Klasen E (1987). A rapid micromethod for apolipoprotein E phenotyping directly in serum. *J Lipid Res* **28**, 455–463.

Hallikainen MA, Sarkkinen ES, Uusitupa MIJ (1999). Effects of low-fat stanol ester enriched margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentration. *Eur J Clin Nutr* **54**, 966–969.

Heinemann T, Kullak-Ublick GA, Pietruck B, von Bergmann K (1991). Mechanisms of action of plant sterols on inhibition of cholesterol absorption. Comparison of sitosterol and sitostanol. *Eur J Clin Pharmacol* **40**, S59–S63.

Hendriks HF, Weststrate JA, van Vliet T, Meijer GW (1999). Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur J Clin Nutr* **53**, 319–327.

Jones PJ, MacDougall DE, Ntanios F, Vanstone CA (1997). Dietary phytosterols as cholesterol-lowering agents in humans. *Can J Physiol Pharmacol* **75**, 217–227.

Koivu-Tikkanen TJ, Ollilainen V, Piironen VI (2000). Determination of phylloquinone and menaquinones in animal products with fluorescence detection after postcolumn reduction with metallic zinc. *J Agric Food Chem* **48**, 6325–6331.

Koivu-Tikkanen TJ, Salo-Väänänen PP, Ollilainen V, Piironen VI (2005). Simultaneous extraction and purification procedure of plasma samples for HPLC determination of vitamins K1, E and A. Submitted.

Law M (2000). Plant sterol and stanol margarines and health. *BMJ* **320**, 861–864.

Lowry OH, Rosenbrough NY, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. *J Biol Chem* **50**, 172–182.

Mensink RP, Ebbing S, Lindbout M, Plat J, van Heugten MMA (2002). Effects of plant stanol esters supplied in low-fat yoghurt on serum

lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations. *Atherosclerosis* **160**, 205–213.

Morton G, Lee S, Buss D, Lawrence P (1995). Intakes and major dietary sources of cholesterol and phytosterols in the British diet. *J Human Nutr Dietet* **8**, 429–440.

Neil HA, Meijer GW, Roe LS (2001). Randomised controlled trial of use by hypercholesterolaemic patients of a vegetable oil sterol-enriched fat spread. *Atherosclerosis* **156**, 329–337.

Nestel P, Cehun M, Pomeroy S, Abbey M, Weldon G (2001). Cholesterol-lowering effects of plant sterol esters and non-esterified stanols in margarine, butter and low-fat foods. *Eur J Clin Nutr* **55**, 1084–1090.

Nissinen M, Gylling H, Vuoristo M, Miettinen TA (2002). Micellar distribution of cholesterol and phytosterols after duodenal plant stanol ester infusion. *Am J Physiol Gastrointest Liver Physiol* **282**, G1009–G1015.

Norman CW, Wong MD (2001). The beneficial effects of plant sterols on serum cholesterol. *Can J Cardiol* **17**, 715–721.

Ovaskainen M-L, Valsta L, Lauronen J (1996). The compilation of food analysis values as a database for dietary studies- the Finnish experience. *Food Chem* **57**, 133–136.

Pelletier X, Belbraouet S, Mirabel D, Mordret F, Perrin JL, Pages X *et al.* (1995). A diet moderately enriched in phytosterols lowers plasma cholesterol concentrations in normocholesterolemic humans. *Ann Nutr Metab* **39**, 291–295.

Phillips KM, Ruggio DM, Bailey JA (1999). Precise quantitative determination of phytosterols, stanols, and cholesterol metabolites in human serum by capillary gas-liquid chromatography. *J Chromatogr B Biomed Sci Appl* **732**, 17–29.

Pietinen P, Hartman AM, Haapa E, Rasanen L, Haapakoski J, Palmgren J *et al.* (1988). Reproducibility and validity of dietary assessment instruments. I. A self-administered food use questionnaire with a portion size picture booklet. *Am J Epidemiol* **128**, 655–666.

Pouteau EB, Monnard IE, Piguet-Welsch C, Groux MJ, Sagalowicz L, Berger A (2003). Non-esterified plant sterols solubilized in low fat milks inhibit cholesterol absorption – a stable isotope doupleblind crossover study. *Eur J Nutr* **42**, 154–164.

Raeini-Sarjaz M, Ntanios FY, Vanstone CA, Jones PJH (2002). No changes in serum fat-soluble vitamin and carotenoid concentrations with the intake of plant sterol/stanol esters in the context of a controlled diet. *Metabolism* **51**, 652–656.

Richelle M, Enslen M, Hager C, Groux M, Tavazzi I, Godin J-P *et al.* (2004). Both free and esterified plant sterols reduce cholesterol absorption and the availability of β-carotene and α-tocopherol in normocholesterolemic humans. *Am J Clin Nutr* **80**, 171–177.

Sugiuchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N *et al.* (1995). Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated alpha-cyclodextrin. *Clin Chem* **41**, 717–723.

Thomsen AB, Hansen HB, Christiansen C, Green H, Berger A (2004). Effect of free plant sterols in low-fat milk on serum lipid profile in hypercholesterolemic subjects. *Eur J Clin Nutr* **58**, 860–870.

Tikkanen MJ, Hogstrom P, Tuomilehto J, Keinanen-Kiukaanniemi S, Sundvall J, Karppanen H (2001). Effect of a diet based on low-fat foods enriched with nonesterified plant sterols and mineral nutrients on serum cholesterol. *Am J Cardiol* **88**, 1157–1162.

Weststrate JA, Meijer GW (1998). Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur J Clin Nutr* **52**, 334–343.

Volpe R, Niittynen L, Korpela R, Sirtori C, Bucci A, Fraone N *et al.* (2001). Effects of yoghurt enriched with plant sterols on serum lipids in patients with moderate hypercholesterolaemia. *Br J Nutr* **86**, 233–239.