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Effects of plant stanol esters supplied in low-fat yoghurt on serum lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations

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Abstract

Oil-based products enriched with plant stanol esters can lower low-density lipoprotein (LDL) cholesterol concentrations by 10–14%. Effectiveness of low-fat products, however, has never been evaluated, although such products fit into a healthy diet. We therefore examined the effects of plant stanol esters emulsified into low-fat yoghurt (0.7% fat) on fasting concentrations of plasma lipids and lipid-soluble antioxidants, which may also change by plant stanol consumption. Sixty non-hypercholesterolemic subjects first consumed daily three cups (3 × 150 ml) of placebo yoghurt for 3 weeks. For the next 4 weeks, 30 subjects continued with the placebo yoghurt, while the other 30 subjects received three cups of experimental yoghurt. Each cup provided 1 g of plant stanols (0.71 g sitostanol plus 0.29 g campestanol) as its fatty acid ester. LDL cholesterol (mean ± S.D.) increased by 0.06 ± 0.21 mmol/l in the placebo group, but decreased by −0.34 ± 0.30 mmol/l in the experimental group. The difference in changes between the two groups of 0.40 mmol or 13.7% was highly significant ($P < 0.001$; 95% confidence interval for the difference, −0.26– −0.53 mmol/l). Effects were already maximal after 1 week. HDL cholesterol and triacylglycerol concentrations did not change. Total tocopherol levels increased by 1.43 μmol/mmol LDL cholesterol (14.0%, $P = 0.015$). β-carotene levels, however, decreased by −0.02 μmol/mmol LDL cholesterol (−14.4%, $P = 0.038$). Decreases in absolute β-carotene concentrations were found in all apoB-containing lipoproteins. LDL-cholesterol standardised phytofluene levels decreased by 21.4 ± 25.7% ($P < 0.001$), while other plasma carotenoid (lutein/zeaxanthin, β-cryptoxanthin, lycopene and α-carotene) levels did not change significantly. We conclude that low-fat yoghurt enriched with plant stanol esters lowers within 1 week LDL cholesterol to the same extent as oil-based products. LDL-cholesterol standardised concentrations of tocopherol increased. The observed decrease in β-carotene levels, as found in many other studies, appears not to be limited to the LDL fraction.

Keywords: Sitostanol; Campestanol; Plant stanols; Yoghurt; Cholesterol; Lipoproteins; Tocopherols; Carotenoids

1. Introduction

The most abundant plant sterols in nature are the 4-desmethyl sterols like sitosterol, campesterol and stigmasterol. Saturation of the double bonds of these sterols results in the formation of plant stanols, such as sitostanol and campestanol. Plant stanols are potent hypocholesterolemic agents and a daily consumption of 2–3 g lowers low-density lipoprotein (LDL) cholesterol concentrations in hyper- and non-hypercholesteromic adults and children by 10–14% without changing high-density lipoprotein (HDL) cholesterol or triacylglycerol concentrations [1–3]. At these intakes, consumption seems to be safe [1–3]. Further, effects are also evident when consumed as part of a prudent diet [4] and in combination with hypocholesterolemic drugs [3]. Foods enriched with these components have therefore a great potential for cholesterol-management.

The chemical structure of plant stanols is almost identical to that of cholesterol. These components can

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therefore compete with both dietary and biliary cholesterol for incorporation into mixed micelles, which results in decreased intestinal cholesterol absorption [5]. Mattson et al. [6] have proposed that dietary fat is the preferred vehicle for supplementation, since fat brings the stanol in its physical state towards the place of action. This may be a reason that so far mainly the hypocholesterolemic effects of plant stanol esters incorporated into oil-based products, such as margarines, shortenings and mayonnaise, have been examined [1]. It may also suggest that plant stanol esters in low-fat products are less effective, but this has never been tested. On the other hand, such low-fat food products would enhance the choice of the consumers and can be part of a recommended, reduced-fat diet. We therefore decided to examine the cholesterol-lowering capacity of plant stanol esters emulsified into a low-fat yoghurt. Effects on fat-soluble antioxidant concentrations in the various lipoproteins were also measured, as plant stanol esters lower plasma antioxidants, even after standardisation for plasma lipid levels [7]. It is not known, however, if these decreases can be explained by reduced concentrations in the LDL fraction or if concentrations in other lipoprotein fractions change as well.

2. Methods

2.1. Subjects

Eighty-six subjects from Maastricht and surrounding areas applied for the study. These subjects were invited for two screening visits to see if they met our eligibility criteria: aged 18–65 years, mean fasting serum total cholesterol concentration < 6.5 mmol/l (251 mg/dl), mean fasting serum triacylglycerol concentration < 3.0 mmol/l (266 mg/dl), body mass index < 30 kg/m^2, diastolic blood pressure < 95 mm Hg, systolic blood pressure < 160 mm Hg, no presence of glucosuria, no use of medication or a diet known to affect serum lipids, no abuse of drugs and/or alcohol, no known allergy or difficulty in digesting milk or dairy proteins, or dietary components in yoghurt, no signs of illness and no history of coronary heart disease. In addition, subjects were not allowed to consume plant stanol esters enriched margarines, to donate blood, or to participate in another biomedical study 30 days before the start and during the study. Finally, pregnant or breast-feeding women were excluded. Seventy-two subjects met these criteria. As the study was designed for only 60 subjects, 16 men and 44 women were selected at random, who all finished the study. All subjects gave their written informed consent before the start of the study.

Subjects were 36 ± 14 years of age (mean $\pm$ S.D.) and had a body mass index of 23.3 ± 2.7 kg/m^2. At the start of the study, mean serum total cholesterol was 5.14 ± 0.78 mmol/l (range: 4.15–6.48 mmol/l) and 5.12 ± 0.80 mmol/l (range: 3.90–6.46 mmol/l) in men and women, respectively. For triacylglycerol, these concentrations were 1.07 ± 0.64 mmol/l (range: 0.48–2.88 mmol/l) and 1.08 ± 0.45 mmol/l (range: 0.38–2.42 mmol/l). Twenty women used oral contraceptives, three women were postmenopausal and seven participants smoked cigarettes.

2.2. Diets and design

This study had a double blind, placebo-controlled, parallel design and was approved by the Medical Ethics Committee of Maastricht University. After a fasting blood sample was taken, subjects were asked to consume daily three cups (150 ml) of placebo yoghurt for a period of 3 weeks. Cups of yoghurt were either consumed with each meal, or one at breakfast and two with dinner. Subjects were also provided with margarines and a shortening based on low erucic acid rapeseed oil (LEAR) to standardise fatty acid intake as much as possible. Subjects were allowed to use these products according to their own preferences and could choose at the start of the study between a margarine with 40% ('light margarine') or with 67% ('regular margarine') absorbable fats. Shortenings contained 98% absorbable fats. It was not allowed to switch between both two types of margarine during the study.

The placebo and experimental yoghurts were similar in colour, taste, absorbable fat content, and caloric value to preserve the double-blind design of the study. At the start of the study subjects could chose between yoghurt with strawberries and/or yoghurt with apricots, but were not allowed to change their choices after the first week. Per 100 g, the yoghurt provided 3.7 g of protein, 12.8 g of carbohydrates, 0.2 g of milk fat and 0.5 g of rapeseed oil fatty acids. Rapeseed oil fatty acids were added to the placebo yoghurt as the experimental yoghurt contained per 100 g 0.67 g of plant stanols esterified with rapeseed oil fatty acids. Plant stanols were added at the expense of water. The plant stanol ester mixtures were obtained by saturation of sitosterol, stigmasterol and campesterol extracted from vegetable oils, giving sitostanol and campestanol. The free plant stanols were then transesterified with rapeseed oil fatty acids (RAISIO GROUP, Raisio, Finland) and mixed with the experimental yoghurt (Senoble, Jouy, France). The stanol ester mixture consisted of 71% sitostanol ester and 29% campestanol ester.

The volunteers had to come at least once a week to the Department to receive a new supply of products. The yoghurt was provided in trays with 24 cups, which included three spare cups, and the margarine and shortening in 1 week portions of 200 g. All left overs at the end of the week had to be returned and were counted

(yoghurt) or weighed (margarine and shortening) to calculate yoghurt, margarine and shortening consumption in that week. The weekly estimated amounts were translated into an estimated daily intake

After the 3 week run-in period, subjects were randomly divided into the two treatment groups. The control group (12 men and 18 women) continued to use the placebo yoghurt for another 4 weeks, while the experimental group (four men and 16 women) used similar yoghurt to which a plant stanol ester mixture was added. As one cup of the experimental yoghurt contained 1 g of plant stanols as its fatty acid ester, targeted daily consumption of plant stanols was 3 g. During the experimental period, all products were coded with a colour label to blind the subjects and the investigators.

Energy and nutrient intakes were estimated by filling in food frequency lists at the end of both periods followed by calculations as described before [15]. Food frequency lists were checked immediately in the presence of the subjects by a registered dietician for incomplete consumption and other deviations. The subjects recorded in diaries any signs of illness, medication used, menstrual phase and any deviations from the protocol. Also, they were required to record the daily amount of yoghurt (and time of consumption), margarine and shortening consumed. Finally, subjects were asked not to change their habitual diet, level of physical exercise, smoking habits, use of alcohol or oral contraceptive intake during the study. Body weight was recorded every week. At the end of the study, subjects had to fill in two questionnaires with items on the gustatory characteristics of the yoghurt and experienced physical inconvenience.

2.3. Blood sampling

Blood was sampled after an overnight fast and after abstinence from drinking alcohol the preceding day and smoking on the morning before blood sampling. All venipunctures were performed by the same person, at the same location and at approximately the same time of the day. No blood was sampled on Mondays. Blood was sampled once at the beginning of the study (day 0) and twice at the end of the run-in period (weeks 2 and 3). During the experimental period, blood samples were taken every week (weeks 4, 5, 6 and 7). To give the volunteers the possibility to check if post-treatment serum lipid and lipoprotein concentrations had returned to pre-treatment values after discontinuation of the intervention, subjects were free to come back to the department for an additional blood sample in weeks 8 and 9. During these last 2 weeks subjects were allowed to eat ad libitum, and no products were provided. Twenty-one subjects from the control group and 14 subjects from the experimental group had blood samples taken at weeks 8 and 9, while 3 subjects from the control group and 11 subjects from the experimental group returned at week 8 only.

A 10 ml clotting tube was sampled at each occasion (CORVAC: integrated serum separator tube, Sherwood Medical Company, St. Louis, USA). Serum was obtained by low-speed centrifugation at $2000 \times g$ for 15 min at 4 °C, at least 1 h after venipuncture, and then immediately stored in small portions at -80 °C. Serum was used for lipids and lipoprotein analysis, for plant sterols and cholesterol precursors, for liver and kidney function parameters, and for C-reactive protein concentrations. At weeks 0, 2, 3, 6 and 7 blood was also sampled using a 10 ml EDTA tube (Sherwood Medical Monoject). In weeks 0, 3 and 7 only, EDTA blood was used for haematological analysis (white blood cells (lymphocytes, monocytes, granulocytes), red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, platelets and mean platelet volume) on a Coulter® Counter (Coulter® MD Series, Coulter Corporation, Hialeah, FL). Then, EDTA plasma was prepared by centrifuging the blood at $2000 \times g$ for 30 min at 4 °C. Aliquots were snap-frozen and stored directly at -80 °C for analysis of antioxidants. In a randomly chosen subset of 12 volunteers (six male and six females from each group), plasma obtained at the end of each dietary period (weeks 3 and 7) was immediately separated into lipoproteins with the following densities (mg/ml): VLDL ($d < 1.006$), IDL ($1.006 < d < 1.019$), LDL ($1.019 < d < 1.055$), HDL_1 plus Lp [a] ($1.055 < d < 1.075$), HDL_2 ($1.075 < d < 1.100$), HDL_3 ($1.100 < d < 1.180$) and a bottom fraction ($d > 1.180$), as described by Terpstra et al. [8]. After separation, fractions were directly stored at -80 °C until analysis.

2.4. Chemical analysis

All serum samples from one subject were analysed in the same analytical run for total cholesterol (CHOD/PAP method; Roche Diagnostics Systems, Hofmann-La Roche Ltd., Basel, Switzerland), HDL cholesterol after precipitation of apoB-containing lipoproteins by adding phosphotungstic acid and magnesium ions (precipitation methods; Monotest cholesterol, Boehringer Mannheim, Mannheim, Germany), and triacylglycerol concentrations (GPO-Trinder; Sigma Diagnostics, St Louis, USA). LDL cholesterol concentrations were calculated using the Friedewald equation [9]. The coefficients of variation within runs were 1.4% for serum total cholesterol, 2.9% for HDL cholesterol and 1.9% for triacylglycerol.

Serum plant sterols and stanols (sitosterol, sitostanol, campesterol, and campestanol), and a cholesterol precursor (lathosterol) were analysed by gas chromatography (GC) as described before [10]. In short, after

saponification with KOH, the non-saponifiable fraction was extracted twice with hexane. Then, the non-saponifiable serum lipids were silylated and the samples were analysed on a GC8000 Top gas chromatograph (Carlo Erba, Milan, Italy) fitted with a 30 m AT-1701 capillary column with an inner diameter of 0.32 mm and a film thickness of 1.0 μm (Alltech, Breda, The Netherlands). 5α-cholestane and 5β-cholestan-3α-ol (Sigma-Alderich Chemie BV, Zwijndrecht, The Netherlands) were added to all serum samples before extraction and used as internal standards for respectively cholesterol and non-cholesterol sterols. Before analysis, serum samples of weeks 2 and 3, and of weeks 6 and 7, were pooled. Samples from one subject were analysed in the same analytical run to exclude variations between runs. A serum pool prepared with blood from healthy volunteers after 4 weeks consumption of plant stanol esters, stored in aliquots at −80 °C, was used as internal control and analysed twice in each run. Coefficients of variation within runs were 3.8% for cholesterol, 8.4% for sitosterol, 4.8% for campesterol, 10.0% for sitostanol, 11.0% for campestanol, and 6.7% for lathosterol.

Plasma concentrations of tocopherols (α-tocopherol, δ-tocopherol, and β + γ-tocopherol), hydrocarbon carotenoids (α-carotene, β-carotene, and lycopene), oxygenated carotenoids (lutein/zeaxanthin and β-cryptoxanthin), phytofluene and retinol were determined in weeks 3 and 7 samples, as described [10]. Briefly, plasma samples were extracted twice with hexane, while retinylacetate was used as internal standard. Antioxidant concentrations were determined by reversed phase high pressure liquid chromatography (HPLC). Pooled samples from one subject of weeks 2 and 3, and of 6 and 7 were analysed in the same analytical run. The mean recovery of retinylacetate was 99 ± 2%.

All separated lipoprotein fractions and the plasma were analysed for cholesterol, triacylglycerol, and lipid-soluble antioxidants. Cholesterol, triacylglycerol and antioxidant concentrations in the lipoprotein fractions were analysed by the same methods as already described. For antioxidants, no internal standards were added to the lipoprotein fractions, but values were re-calculated to a 100% recovery. All samples from one subject were analysed in the same analytical run.

Liver function (ALAT, ASAT, γ-GT, and C-RP) and kidney function (creatinine) were analysed in samples from weeks 0, 3, and 7, as described [11].

3. Statistics

The data were analysed with Statview 4.5 [12]. Before the start of the study it was calculated that the power to detect a true difference of 0.30 mmol/l for LDL cholesterol between treatments was 90%. For each subject, lipid and lipoprotein concentrations of weeks 2 and 3 (run-in period), and of weeks 6 and 7 (experimental period) were first averaged. Changes for all parameters were calculated for each subject as the difference between values of the experimental and run-in periods. The differences in changes between the groups were tested with an unpaired T-test.

4. Results

4.1. Dietary intakes, yoghurt consumption, body weight and safety parameters

Table 1 shows that energy intake and the proportions of energy from fat, fatty acids, carbohydrates and protein, as well as cholesterol and fibre intake were essentially similar in the two groups during both the run-in and the experimental periods.

During both the run-in period and the experimental period the mean estimated daily consumption of yoghurt was 3.0 cups (range: 2.9–3.0 cups) in both groups. For the experimental group only, this resulted in a supplemented intake of 2.98 g of total plant stanols provided as its fatty acid ester (range: 2.86–3.00 g), of which 2.11 g were sitostanol (range: 2.03–2.12 g) and 0.87 g campestanol (range: 0.83–0.87 g) during the experimental period.

Changes in body weight of the subjects during the experimental period were small and were 0.2 ± 1.0 kg for the control group and −0.1 ± 0.8 kg in the experimental group ($P = 0.175$ for the difference in changes between the two groups). Values for ALAT, ASAT, γ-GT, creatinine and C-RP, and for haematological parameters did not change from the start until the end of the study (data not shown). Inspection of the diaries did not reveal any serious deviations from the protocol. There were no suggestions that consumption of the yoghurt with plant stanol esters caused any physical complaints. The yoghurt with strawberries and the yoghurt with apricots were both very well appreciated. When subjects were asked what type of yoghurt they had consumed during the study, 40% of the subjects from the control group and 20% of the subjects from the experimental group identified the type of yoghurt correctly. This demonstrates that blinding was successful.

4.2. Serum lipids and lipoproteins

Mean changes in serum lipid and lipoprotein concentrations are shown in Table 2. During the experimental period serum total cholesterol concentrations increased slightly from 4.76 to 4.83 mmol/l in the control group, but decreased from 4.98 to 4.62 mmol/l in the experimental group. The difference in changes between the two

groups of 0.43 mmol or 8.7% was highly significant ($P < 0.001$ for the difference in absolute changes; 95% confidence interval (CI), -0.29– -0.57 mmol/l). LDL cholesterol concentrations, as calculated with the Friedewald-equation [9], increased by 0.06 mmol/l in the control group, but decreased by -0.34 mmol/l in the experimental group. Again, the difference in changes between the two groups of 0.40 mmol or 13.7% was highly significant ($P < 0.001$; 95% CI for the difference, -0.26– -0.53 mmol/l). HDL cholesterol concentrations did not change ($P = 0.403$; 95% CI for the difference, -0.08–0.03 mmol/l). As a consequence, the total to HDL cholesterol ratio hardly changed in the control group, but decreased by -0.28 in the experimental group ($P < 0.001$; 95% CI for the difference, -0.17– -0.44). Triacylglycerol concentrations remained essentially unchanged during the study ($P = 0.633$; 95% CI for the difference, -0.16–0.10 mmol/l). Relative concentrations of serum LDL cholesterol during the study are shown in Fig. 1. Effects of plant stanol ester consumption on total and LDL cholesterol concentrations were already maximal after 1 week, while values between the two groups were comparable 2 weeks after discontinuation of the experimental yoghurt.

Table 1
Daily intake of energy and nutrients during the study

	Control group	Experimental group
Energy (MJ)		
Run-in	11.6 ± 2.5	10.1 ± 2.2
Experimental	11.3 ± 2.5	9.5 ± 2.0
Change	−0.3 ± 1.3	−0.7 ± 1.4
Fat (energy %)		
Run-in	31.3 ± 4.7	29.4 ± 4.8
Experimental	31.7 ± 3.5	29.1 ± 5.9
Change	0.3 ± 2.6	−0.4 ± 2.8
SAFA (energy %)		
Run-in	11.1 ± 2.0	10.9 ± 2.0
Experimental	11.4 ± 1.6	10.8 ± 2.5
Change	0.2 ± 1.2	−0.1 ± 1.2
MUFA (energy %)		
Run-in	12.1 ± 2.3	11.1 ± 2.1
Experimental	12.2 ± 1.8	10.9 ± 2.5
Change	0.1 ± 1.1	−0.2 ± 1.3
PUFA (energy %)		
Run-in	5.8 ± 1.0	5.3 ± 1.1
Experimental	5.9 ± 0.8	5.3 ± 1.4
Change	0.0 ± 0.7	0.0 ± 0.8
Linoleic acid (energy %)		
Run-in	4.7 ± 0.9	4.3 ± 0.9
Experimental	4.7 ± 0.8	4.3 ± 1.2
Change	0.0 ± 0.7	0.0 ± 0.6
α-Linolenic acid (energy %)		
Run-in	0.8 ± 0.2	0.7 ± 0.2
Experimental	0.8 ± 0.2	0.7 ± 0.2
Change	0.0 ± 0.1	0.0 ± 0.1
Protein (energy %)		
Run-in	14.7 ± 2.0	15.6 ± 2.4
Experimental	14.9 ± 2.0	16.1 ± 2.3
Change	0.2 ± 1.1	0.5 ± 1.2
Carbohydrates (energy %)		
Run-in	50.8 ± 5.0	52.9 ± 4.8
Experimental	50.1 ± 5.0	52.6 ± 6.0
Change	−0.7 ± 3.0	−0.3 ± 3.1
Cholesterol (mg/MJ)		
Run-in	20.1 ± 4.5	19.6 ± 5.4
Experimental	19.9 ± 4.5	20.3 ± 5.8
Change	−0.2 ± 3.1	0.6 ± 3.9
Fibre (g/MJ)		
Run-in	2.4 ± 0.5	2.6 ± 0.6
Experimental	2.4 ± 0.4	2.6 ± 0.5
Change	0.0 ± 0.3	0.1 ± 0.4

Values refer to 30 subjects in each group and are means ± S.D. SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2
Effects of a yoghurt enriched with plant stanol esters on serum lipid and lipoprotein concentrations during the study

	Control group	Experimental group
Total cholesterol (mmol/l)		
Run-in	4.76 ± 0.81	4.98 ± 0.81
Experimental	4.83 ± 0.80	4.62 ± 0.78
Change	0.07 ± 0.23	−0.36 ± 0.31[a]
LDL cholesterol (mmol/l)		
Run-in	2.86 ± 0.87	2.92 ± 0.87
Experimental	2.92 ± 0.87	2.68 ± 0.74
Change	0.06 ± 0.21	−0.34 ± 0.29[a]
HDL cholesterol (mmol/l)		
Run-in	1.43 ± 0.32	1.45 ± 0.36
Experimental	1.44 ± 0.32	1.43 ± 0.33
Change	0.01 ± 0.11	−0.01 ± 0.10
Total to HDL cholesterol ratio		
Run-in	3.55 ± 1.21	3.67 ± 1.12
Experimental	3.58 ± 1.24	3.39 ± 0.97
Change	0.03 ± 0.27	−0.28 ± 0.26[a]
Triacylglycerol (mmol/l)		
Run-in	1.02 ± 0.52	1.13 ± 0.42
Experimental	1.03 ± 0.50	1.11 ± 0.43
Change	0.01 ± 0.15	−0.02 ± 0.31

Values refer to 30 subjects in each group and are means ± S.D.
[a] $P < 0.001$ for the difference in changes between the experimental group and the control group.

4.3. Plant sterols, stanols and cholesterol precursors

As expected, consumption of the yoghurt supplemented with plant stanol esters lowered intestinal cholesterol absorption, as indicated by the decreases in

cholesterol-standardised sitosterol ($P < 0.001$) and campesterol concentrations ($P < 0.001$; Table 3). Plasma cholesterol-standardised sitostanol ($P < 0.001$) and campestanol ($P = 0.035$) concentrations, however, increased. To compensate for the decrease in intestinal cholesterol absorption, endogenous cholesterol synthesis, as indicated by the increase in plasma cholesterol-standardised lathosterol concentrations, increased ($P = 0.017$).

4.4. Antioxidant concentrations

Consumption of the plant stanol esters lowered significantly plasma concentrations of the various carotenoids and of phytofluene, while concentrations of the different tocopherol isomers and of retinol remained unchanged (data not shown). After standardisation of the lipid-soluble antioxidants for LDL-cholesterol concentrations (Table 4), the decrease in β-carotene of -0.02 ± 0.04 μmol/mmol LDL cholesterol ($-12.9 \pm 21.2\%$) in the experimental group was significantly different from the change of 0.00 ± 0.02 μmol/mmol LDL cholesterol ($1.5 \pm 24.4\%$) in the control group ($P = 0.038$; 95% CI for the difference 0.00–-0.03 μmol/mmol LDL cholesterol). LDL-cholesterol standardised phytofluene levels were significantly decreased by $21.4 \pm 25.7\%$ ($P < 0.001$). In contrast, plant stanol ester consumption increased LDL-cholesterol standardised concentrations of the various tocopherols. For δ-tocopherol the difference between the two groups was 0.02 μmol/mmol LDL cholesterol or 31.4% ($P = 0.031$; 95% CI for the difference, 0.00–0.03 μmol/mmol LDL cholesterol), for β + γ-tocopherol 0.26 μmol/mmol LDL cholesterol or 27.9% ($P = 0.011$; 95% CI for the difference, 0.06–0.45 μmol/mmol LDL cholesterol), and for α-tocopherol 1.16 μmol/mmol LDL or 12.7% ($P = 0.021$; 95% CI for the difference, 0.18–2.14 μmol/mmol LDL cholesterol).

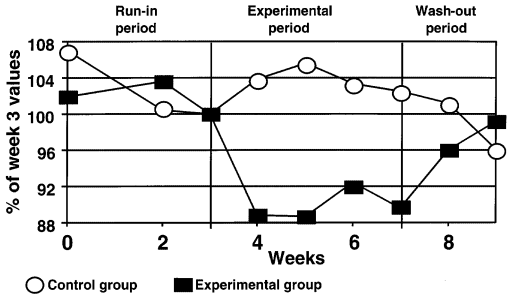

Fig. 1. Relative concentrations of serum LDL cholesterol during the study. Concentrations in week 3 were set at 100%. Values from weeks 0–7 refer to 30 subjects in each group. Twenty-one subjects from the control group and 14 subjects from the experimental group had blood samples taken at both weeks, while three subjects from the control group and 11 subjects from the experimental group returned at week 8 only.

Table 3
Cholesterol-standardised serum non-cholesterol sterol concentrations during the study

	Placebo group	Experimental group
Sitosterol ($10^2 \times$ μmol/mmol cholesterol)		
Run-in	172.6 ± 67.7	159.8 ± 61.1
Experimental	164.1 ± 57.1	109.6 ± 39.6
Change	-8.5 ± 25.0	-50.2 ± 31.8[b]
Campesterol ($10^2 \times$ μmol/mmol cholesterol)		
Run-in	397.5 ± 141.0	367.5 ± 143.4
Experimental	405.1 ± 136.9	254.0 ± 98.9
Change	7.7 ± 49.9	-113.5 ± 75.9[b]
Sitostanol ($10^2 \times$ μmol/mmol cholesterol)		
Run-in	11.6 ± 9.9	12.5 ± 10.2
Experimental	10.9 ± 9.0	19.5 ± 13.6
Change	-0.8 ± 4.6	7.1 ± 7.3[b]
Campestanol ($10^2 \times$ μmol/mmol cholesterol)		
Run-in	10.9 ± 5.7	11.0 ± 5.7
Experimental	9.7 ± 4.2	12.7 ± 6.4
Change	-1.3 ± 5.4	1.7 ± 5.1[a]
Lathosterol ($10^2 \times$ μmol/mmol cholesterol)		
Run-in	119.2 ± 63.9	106.4 ± 58.6
Experimental	115.3 ± 64.4	120.0 ± 62.8
Change	-3.9 ± 26.1	13.5 ± 28.9[a]

Values refer to 30 subjects in each group and are means ± S.D.
[a] $P < 0.05$ for the difference in changes between the experimental group and the control group.
[b] $P < 0.001$ for the difference in changes between the experimental group and the control group.

When antioxidant concentrations were standardised for total cholesterol or for total cholesterol plus triacylglycerol, effects were essentially similar, although for most carotenoids the differences in changes reached statistical significance. In general, changes were negative for the hydrocarbon carotenoids (i.e. β-carotene, α-carotene and lycopene), slightly negative or zero for the oxygenated carotenoids (lutein/zeaxanthin, β-cryptoxanthin), and positive for the tocopherols, irrespective of the method of standardisation.

In three women and in three men for each group, antioxidant concentrations were determined in the various lipoprotein fractions. For these 12 subjects, changes in absolute total tocopherol concentrations were -0.49 ± 3.51 μmol/l in the control group and 3.24 ± 6.11 μmol/l in the experimental group ($P = 0.224$). Also, concentrations of total tocopherols in the various lipoproteins were not significantly different between the two groups (Fig. 2). The increase in the experimental

Table 4
LDL cholesterol-standardized lipid-soluble antioxidant concentrations during the study

	Placebo group	Experimental group
Total tocopherols (μmol/mmol LDL cholesterol)		
Run-in	11.33 ± 3.42	10.31 ± 2.29
Experimental	11.87 ± 2.99	11.27 ± 3.07
Change	−0.46 ± 1.65	0.97 ± 2.60[a]
δ-Tocopherol (μmol/mmol LDL cholesterol)		
Run-in	0.05 ± 0.03	0.04 ± 0.01
Experimental	0.04 ± 0.02	0.05 ± 0.03
Change	−0.01 ± 0.02	0.01 ± 0.03[a]
β+γ-Tocopherol (μmol/mmol LDL cholesterol)		
Run-in	1.01 ± 0.46	0.75 ± 0.19
Experimental	0.94 ± 0.47	0.94 ± 0.47
Change	−0.07 ± 0.33	0.19 ± 0.40[a]
α-Tocopherol (μmol/mmol LDL cholesterol)		
Run-in	10.27 ± 3.02	9.52 ± 2.28
Experimental	9.89 ± 2.62	10.29 ± 2.75
Change	−0.39 ± 1.40	0.77 ± 2.25[a]
Oxygenated carotenoids (μmol/mmol LDL cholesterol)		
Run-in	0.33 ± 0.12	0.38 ± 0.20
Experimental	0.32 ± 0.12	0.36 ± 0.19
Change	−0.01 ± 0.06	−0.02 ± 0.09
Lutein/Zeaxanthin (μmol/mmol LDL cholesterol)		
Run-in	0.24 ± 0.10	0.27 ± 0.16
Experimental	0.23 ± 0.10	0.27 ± 0.15
Change	−0.01 ± 0.06	−0.01 ± 0.07
β-Cryptoxanthin (μmol/mmol LDL cholesterol)		
Run-in	0.09 ± 0.04	0.11 ± 0.05
Experimental	0.08 ± 0.03	0.10 ± 0.05
Change	−0.01 ± 0.01	−0.01 ± 0.03
Hydrocarbon carotenoids (μmol/mmol LDL cholesterol)		
Run-in	0.30 ± 0.12	0.35 ± 0.17
Experimental	0.29 ± 0.11	0.32 ± 0.14
Change	−0.01 ± 0.05	−0.03 ± 0.08
Lycopene (μmol/mmol LDL cholesterol)		
Run-in	0.18 ± 0.10	0.19 ± 0.09
Experimental	0.18 ± 0.08	0.18 ± 0.08
Change	−0.01 ± 0.05	−0.01 ± 0.06
α-Carotene (μmol/mmol LDL cholesterol)		
Run-in	0.01 ± 0.01	0.02 ± 0.01
Experimental	0.01 ± 0.01	0.02 ± 0.01
Change	0.00 ± 0.00	0.00 ± 0.01
β-Carotene (μmol/mmol LDL cholesterol)		
Run-in	0.10 ± 0.05	0.14 ± 0.11
Experimental	0.10 ± 0.05	0.12 ± 0.10
Change	0.00 ± 0.02	−0.02 ± 0.04[a]
Phytofluene (mV × min/mmol LDL cholesterol; amplification 100)		
Run-in	0.12 ± 0.06	0.15 ± 0.11
Experimental	0.11 ± 0.08	0.11 ± 0.07
Change	−0.01 ± 0.04	−0.04 ± 0.05[b]

Values refer to 30 subjects in each group and are means ± S.D. Total tocopherols are the sum of δ-tocopherol plus β+γ-tocopherol plus α-tocopherol concentrations; oxygenated carotenoids are the sum of lutein/zeaxanthin plus β-cryptoxanthin concentrations; and hydrocarbon carotenoids are the sum of lycopene plus α-carotene plus β-carotene concentrations.

[a] $P < 0.05$ for the difference in changes between the experimental group and the control group.

[b] $P < 0.01$ for the difference in changes between the experimental group and the control group.

group can largely be explained by the results from one subject, whose tocopherol concentration increased by 15 μmol/l during the study. When this subject was excluded from the statistical analyses, results between the two groups were even more comparable (data not shown). Concentrations of the oxygenated carotenoids were also not significantly different between the two groups. It should be mentioned, however, that for the whole study population absolute total plasma concentrations of the oxygenated carotenoids were significantly decreased in the experimental group by −0.14 μmol/l ($P < 0.001$). For the more lipophylic hydrocarbon carotenoids the change in plasma concentrations of −0.01 ± 0.10 μmol/l in the control group tended to be less than the decrease of −0.18 ± 0.19 μmol/l in the experimental group ($P = 0.073$). The differences in changes between the two groups for the VLDL fraction reached statistical significance ($P = 0.034$). For IDL and LDL decreases were also more pronounced in the experimental group, although no statistically significant treatment effects could be observed.

5. Discussion

This double-blind study in 60 healthy volunteers showed that a low-fat yoghurt enriched with plant stanol esters lowered serum LDL cholesterol concentrations by nearly 14%. Effects were already maximal after 1 week. Daily intake of plant stanols, which were provided as fatty acid esters, was 3 g. Because serum HDL cholesterol concentrations were not affected, the total to HDL cholesterol ratio was decreased by 8%,

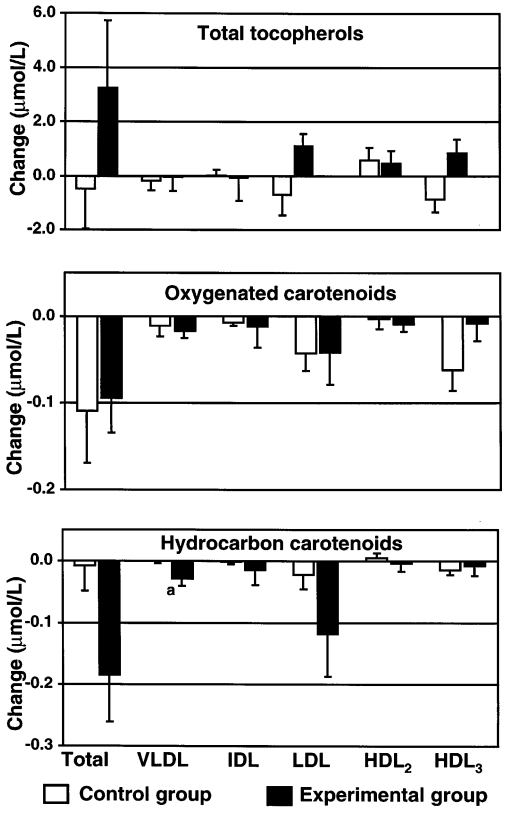

Fig. 2. Changes (mean ± SEM) in concentrations of total tocopherols, oxygenated carotenoids (lutein/zeaxanthin and β-cryptoxanthin), and hydrocarbon carotenoids (α-carotene, β-carotene, and lycopene) during the study. $^{a}P < 0.05$ for the difference in changes between the experimental group and the control group.

while no effects on serum triacylglycerol concentrations were found. Compliance to the test products was not only confirmed by the reported yoghurt consumption, but also by the changes in plasma stanol and sterol concentrations, which were as anticipated [10].

Law has estimated that at intakes of more than 2 g plant stanols per day (range 2.0–4.0 g) the average reduction in LDL cholesterol may depend on age and varies between 9 and 14% [1]. The decrease in LDL cholesterol of about 14% in our study agrees very well with this estimate. [1]. Our study, however, differs from other studies in that plant stanol esters were ingested in a product with a very low-fat content (0.7%), while in other studies oil-based products, such as margarine and mayonnaise, were used. This demonstrates that the ability of plant stanol esters to block intestinal cholesterol absorption is not necessarily compromised by a low-fat food matrix. The diet of our subjects was also low in fat (30% of energy) and its nutrient composition closely resembled that of a recommended diet. Therefore, it seems that-as for low-fat, plant stanol-ester containing margarines [4]-this yoghurt is effective when used as part of a prudent diet.

It is not known if our results can be extrapolated to low-fat products enriched with free plant stanols. In fact, Ostlund et al. [13] have reported that a single dose of 1000 mg of free sitostanol powder, provided as a capsule, lowered the relative intestinal cholesterol absorption by about 11%. Effects of only 300 and 700 mg of free sitostanol, however, incorporated into lecithin micelles were about three times as potent. This may suggest that for free plant stanols the food matrix or the background diet is of more importance than for esterified plant stanols.

The Friedewald-equation has been used in most studies to estimate effects of plant stanol or sterol esters on LDL-cholesterol concentrations [1]. This is a well-validated and generally accepted method, but it should be realised that the density range used to define LDL by Friedewald et al. [9] includes IDL, which contributes in general less than 5% to the plasma total cholesterol level. Analysis of IDL cholesterol concentrations, however, may provide insight into possible mechanisms. Gylling et al. [14] have now reported that plant stanol ester consumption lowered not only LDL-cholesterol, but also, slightly, IDL cholesterol. Our results from the analysis of the various lipoproteins also suggested that IDL cholesterol was decreased to some extent. The decrease in IDL cholesterol may, at least partly, be explained by our recent finding that plant stanol ester consumption increases LDL-receptor protein on mononuclear cells [15], which not only clears LDL from the circulation but also IDL.

Several studies have examined the effects of plant stanol and sterol esters on fat-soluble antioxidants. As recently reviewed [10], all studies found decreases in absolute α-plus β-carotene levels, although the changes did not always reach statistical significance. Levels of the oxygenated carotenoids (lutein/zeaxanthin and β-cryptoxanthin) were also lowered [7], but those of retinol not [10]. Our present results are in agreement with these other studies [7,10]. In contrast with other studies [10], however, absolute tocopherol concentrations were not changed. Whether this is due to the differences in the food matrix remains to be determined. Because plasma lipid-soluble antioxidants are transported by lipoproteins, a decrease in plasma lipids may simply be the cause of the decreased plasma lipid-soluble antioxidant concentrations. Therefore, concentrations are generally standardised for a plasma lipid fraction, but no uniformity exists [7,10,16]. We chose to standardise for plasma concentrations of LDL-cholesterol, because it is mainly this lipoprotein fraction which is lowered by plant stanol ester consumption. If the proportional decrease in plasma antioxidant concentrations is similar to that in LDL cholesterol, then no effect on LDL-cholesterol standardised antioxidant concentrations is to be expected. This, however, was not the case. After standardisation for LDL cholesterol,

levels of the various tocopherols were significantly increased, those of the various oxygenated carotenoids were unchanged, while those of the β-carotene – a hydrocarbon carotenoid-were decreased. This may suggest that changes in antioxidant concentrations can not simply be explained by a decrease in the number of circulating LDL particles. Indeed, analyses of antioxidant concentrations in a subset of the participants showed that changes in total tocopherol concentrations in the various lipoproteins were not significantly different between the control and experimental groups. As already discussed, LDL cholesterol was significantly decreased in the experimental group, which explains the increased total tocopherol to LDL-cholesterol in the experimental group (Table 4). In fact, this does suggest that the LDL particle became relatively enriched in tocopherols after consumption of the experimental yoghurt.

Concentrations of the hydrocarbon carotenoids decreased significantly in the VLDL, and also in the IDL and LDL fractions, but hardly in the HDL density range. Earlier we had reported that on plant stanol-ester rich diets the decrease in the hydrocarbon carotenoids, which are more hydrophobic than the oxygenated carotenoids, was partly related to the decrease in cholesterol absorption [10]. This suggests that plant stanol esters may lower the amount of the hydrocarbon carotenoids incorporated into the chylomicron fraction. For β-carotene it has been reported that a decreased carriage in chylomicrons also results in reduced levels in VLDL [17]. This may also explain for our study the reduced levels in IDL and LDL, for which VLDL is the precursor. The precise mechanisms by which plant stanol or sterol esters lower plasma carotenoid concentrations, as well as the possible health implications in the longer term, remain to be determined. Also our results on the concentrations of the oxygenated carotenoids in the various lipoproteins are inconclusive, as changes in the subset of twelve people were not representative of the whole study population.

To summarise, our findings suggest that within 1 week a low-fat yoghurt enriched with plant stanol esters lowers LDL cholesterol concentrations to the same extent as oil-based products. The reduction in absolute plasma tocopherol concentrations, as found in many other studies, was not observed in this study. In fact, our results suggested the LDL particle became enriched in tocopherols. The frequently reported decrease in carotenoids, and especially of the hydrocarbon cartenoids, has been confirmed in this study and appears not be limited to the LDL fraction.

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