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ORIGINAL CONTRIBUTION

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Plant sterol ester-enriched milk and yoghurt effectively reduce serum cholesterol in modestly hypercholesterolemic subjects*

Summary *Background* The cholesterol-lowering efficacy of plant sterol esters (PSteE) or stanol esters (PStaE) in regular- and low-fat spreads has been consistently demonstrated, while their effectiveness in a low-fat, aqueous food carrier such as milk and yoghurt is less well established. *Aim of the study* Two studies were carried out to assess the cholesterol-lowering effect of PSteE-enriched low-fat milk and PSteE- and PStaE-enriched low-fat yoghurt in modestly hypercholesterolemic subjects (total cholesterol between 5–7.5 mmol/l). *Methods* Study one was a single blind crossover design with 4 phases of 3-week interventions. Subjects consumed 300 ml/d of placebo or PSteE-milk (2.0 g plant sterols/d) alone or combined with 25 g/d of placebo or PSteE-spread. Study two was a fully randomised, double blind crossover design with 3 phases of 3-week interventions. Subjects consumed 2 portions (150 g tubs each) of placebo, PSteE-yoghurt (1.8 g plant sterols/d) or PStaE-yoghurt (1.7 g plant stanols/d). In study one 39 subjects (21 men and 18 women) and in study two 40 subjects (17 men and 23 women) completed the dietary intervention. *Results* In study one, PSteE-milk and PSteE-spread were equally efficacious in lowering total and LDL-cholesterol as compared to placebo by 6–8 % and 8–10 %, respectively. No significant additional cholesterol-lowering was observed with the combination of PSteE-milk and PSteE-spread (4 g plant sterols/d). PSteE-enriched milk and the combination of PSteE-enriched milk plus spread both lowered lipid-adjusted serum β-carotene concentrations by 10–14 % ($P < 0.02$), while the PSteE-rich spread alone did not significantly alter serum β-carotene levels. In study two, the PSteE- and PStaE-enriched yoghurts reduced LDL-cholesterol significantly compared to placebo by 0.27 ± 0.05 mmol/l (6 %) and 0.23 ± 0.05 mmol/l (5 %), respectively. In both studies, there was no effect on HDL-cholesterol and triacylglycerol concentrations. *Conclusion* Plant sterols in the form of their esters when provided in low-fat milk and yoghurt are effective in lowering total and LDL-cholesterol.

Key words modestly hypercholesterolemic subjects – milk – yoghurt – plant sterols – low-fat foods

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Abbreviations

BMI	body mass index
PSteE	plant sterol esters
PStaE	plant stanol esters
TC	total cholesterol
LDL	low density lipoprotein
HDL	high density lipoprotein

Introduction

Plant sterols (or phytosterols) are natural compounds structurally very similar to cholesterol, which are found ubiquitous in plant foods. To date, numerous studies have shown that dietary plant sterols, especially in the form of plant sterol esters (PSteE) effectively lower total (TC) and low-density (LDL) cholesterol when incorporated into fat-based foods such as margarine and spreads [1–7]. In a meta-analysis by Law [8] it was reported that at a dose of ≥ 2 g/d of plant sterols or stanols (the saturated form), the average reduction in serum LDL-cholesterol is 0.33 to 0.54 mmol/l or 9 to 14 %, depending on the age of the participants studied. A more recent meta-analysis including 41 trials confirmed these findings and concluded that 2 g/d of plant sterols or stanols lowers LDL-cholesterol by 10 % [7].

As plant sterols or stanols, due to their lipophilic nature, have mostly been added to margarine and spreads, fewer studies have examined the efficacy of plant sterols in low-fat or even non-fat, aqueous-type food carriers such as bread, cereals, yoghurt or milk [9–13]. As dietary guidelines recommend limiting total fat intake to around 30 % of total energy intake, fat-based products may not always be the preferred choice for health-conscious consumers. In fact, consumer surveys have shown that the amount of PSteE-enriched spreads consumed even by frequent users is well below the required intake to achieve the desirable dose of 2 g/d of plant sterols for maximal LDL-cholesterol reduction [14].

Low-fat dairy products such as milk and yoghurt are healthy and acceptable foods for the consumer [15–19]. Thus, incorporating plant sterols into low-fat dairy products would offer additional dietary options to maintain a healthy cholesterol concentration or to maximize the effectiveness of a cholesterol-lowering treatment.

Nestel et al. [12] observed that PSteE and non-esterified, free stanols, of which two-thirds were incorporated into low-fat foods including breakfast cereal and bread, lowered LDL-cholesterol by 13.6 %. A recent study showed that consumption of a low-fat yoghurt (0.7 % total fat) providing 3 g/d of plant stanols in the form of plant stanol esters (PStaE) lowered LDL-cholesterol by 13.7 % compared to the placebo group [11]. In addition, plant sterols incorporated into a low-fat yoghurt-based drink have also been shown to be effective in lowering cholesterol. A daily intake of 1 g of plant sterols resulted in a decrease in TC and LDL-cholesterol of 7 % and 11 % respectively; however when taking the reduction seen in the placebo group into account, the net effect was 4.4 % for TC and 6.2 % for LDL-cholesterol [10].

A very recent paper by Jones et al., however, showed no effect in lowering TC and LDL-cholesterol when consuming plant sterol-enriched low-fat or non-fat beverages [20] suggesting that efficacy is not always achieved with such food formats. It needs to be noted that Jones et al. [20] studied the effect of free plant sterols, whereas in the study of Mensink et al. [11] the stanols are given in their esterified form as PStaE. Moreau et al. [21] and others have suggested that, at least with free plant sterols or stanols, the efficacy in lowering LDL-cholesterol is dependent on the physical form of the plant sterol formulation, the food format, and possibly the fat content of the food. This may possibly not apply for PSteE or PStaE as esterification with dietary fatty acids increases fat-solubility, which aids their incorporation into a variety of different food matrices. The number of well-designed studies, which have assessed the efficacy of PSteE or PStaE in foods other than spreads and margarine is, however, still relatively small.

Therefore, the objectives of these studies were a) to assess the effects of PSteE-enriched low-fat milk and low-fat yoghurt on serum lipids in two separate dietary intervention trials, b) to study the cholesterol-lowering effects of PSteE in milk alone and in combination with a PSteE-enriched spread, c) to examine the effects on serum carotenoids as a consequence of plant sterol intake and d) to compare the cholesterol-lowering effects of a low-fat yoghurt fortified with PSteE versus PStaE.

Subject and methods

Two studies were carried out, termed study one and study two, each with 3-week intervention periods.

Subjects

Forty (study one) and forty-two (study two) modestly hypercholesterolemic men and women were recruited by public advertisements and were screened on the basis of the following selection criteria: aged between 20–75 years with fasting total cholesterol between 5.0 and 7.5 mmol/l. Subjects with marked obesity (BMI > 31 kg/m^2), a fasting triglyceride concentration greater than 4.5 mmol/l and diabetes mellitus (as assessed from a medical questionnaire), or taking supplements (e. g. fish oil) likely to affect lipid parameters were excluded. Subjects consuming plant sterol enriched foods were permissible provided they had at least a 3-week washout prior to study commencement. In study one, one subject withdrew prior to the study commencement and none dropped out during the study, hence 21 men and 18 women completed the study. In study two, two subjects withdrew prior to commencement and all remaining 40 subjects (17 men and 23 women) completed the study. Baseline characteristics of the subjects who completed the intervention are presented in Table 1. The study protocols were approved by the CSIRO Health Sciences and Nutrition Human Ethics Committee. All subjects gave

Table 1 Baseline characteristics of the subjects in study 1 and 2

	Study 1	Study 2
Number of subjects	39	40
Gender (male/female)	21/18	17/23
Age, years	51.5±11.2	60.4±7.1
BMI, kg/m^2	25.9±2.1	26.5±3.2
Total cholesterol, mmol/l	6.8±0.82	6.52±0.87
LDL-cholesterol, mmol/l	4.83±0.79	4.48±0.75
HDL-cholesterol, mmol/l	1.26±0.39	1.42±0.43

Data are presented as means ± SD

their informed written consent before the start of the study.

Experimental design and diets

Study one was a single blind, partially randomised, placebo controlled, crossover study, which consisted of 4 intervention periods each of 3-week duration, giving a total study period of 12 weeks. Participants were asked to maintain their usual dietary habits throughout the study. The 4 interventions were as follows: 300 ml/d of a low-fat milk with or without 2 g of plant sterols (delivered as PSteE) combined with 25 g/d of spread with or without 2 g of plant sterols (delivered as PSteE). In treatment sequence 1, plant sterol-free milk was used in the first two treatment periods and PSteE-enriched milk in the last two, while in sequence 2 this was reversed. In sequence 1, the order of spread intake in both milk phases was plant sterol-free and then with PSteE, while in sequence 2 this was reversed.

All test and control food products were supplied by Unilever Bestfoods and portion packed in a coded form. The spread was a regular PSteE-enriched margarine as available on the Australian market containing 64% fat and 8% plant sterols (13% PSteE). The PSteE-enriched milk provided per 100 g, 3.2% protein and 1.4% fat, of which 0.9% was dairy fat with the remaining coming from the fatty acid part of the PSteE. The PSteE consisted of vegetable oil-based sterols esterified with sunflower oil fatty acids and contained 47% sitosterol, 27% campesterol, 16% stigmasterol with other plant sterols contributing less than 5% of the total sterol mixture.

Study two was a fully randomised, placebo controlled, and double blind crossover study design with 3-week intervention periods giving a total study period of 9 weeks. In this study the effects on serum lipids of PSteE-enriched (1.8 g plant sterols/d) and PStaE-enriched (1.7 g plant stanols/d) low-fat yoghurt were compared to a control yoghurt. Subjects consumed daily in total 300 g low-fat yoghurt provided as two cups (150 g). The control and test yoghurts were similar in colour, taste and energy value. Per 100 g, the PSteE- and PStaE-enriched yoghurts contained 4.3 g of protein, 13.1 g of carbohydrates and 0.54 of total fat, of which 0.1 g was from dairy fat.

Washout periods between the interventions were not considered necessary as plant sterols and stanols are only minimally absorbed and since 3–4 weeks is generally considered adequate to reach a new steady-state condition regarding serum cholesterol, triacylglycerol and HDL cholesterol concentrations.

The subjects consumed the control and test products as a part of their normal diet and were advised not to make any dietary changes during the study. For both the studies, food checklists were completed daily. In study one, nutrient intakes were calculated based on weighed food records, which were completed on 3 consecutive days (Sunday, Monday, and Tuesday), every 3 weeks; the specific dates were noted on the study calendar given to all subjects. Nutrient intakes were analysed with DIET/1 nutrient calculation software (Xyris Software, Highgate Hill, Australia), a computer database of foods in which nutrient composition is based on that of Australian foods with modifications to include both data from commercial sources and from analyses of the spreads.

In study two, nutrient intakes were assessed at the end of each 3-week intervention using the Anti Cancer Council of Victoria food frequency questionnaire which has been validated against 7 day weighed food records [22]. The subjects' weights and diets were monitored, checklists collected and new checklists re-issued every 2–3 weeks by the dietician (study one) of the clinical trial manager (study two). Compliance was assessed by asking subjects whether they had missed eating the yoghurts on any of the intervention days as well as noting the records from the checklists.

Methods

In study one, fasting blood samples for serum lipids and plasma carotenoids were performed on 2 consecutive days at the end of each intervention phase. Full routine clinical biochemistry including glucose, magnesium and γ-GT and vitamin D, haematology, coagulation and dipstick urinalysis was performed by an accredited clinical pathology laboratory. In study two, fasting blood samples were collected once a week with 10 samples in total.

Serum lipids

In both studies, venous blood samples (20 ml) were taken into plain tubes from fasted subjects (12 h). Serum was separated by low-speed centrifugation at 600 g for 10 min at 5 °C (GS-6R centrifuge; Beckman, Fullerton, CA) and frozen at –20 °C. At the end of the study, all sam-

ples from each subject were analysed within the same analytic run to minimise inter-assay variation. Total cholesterol [23] and triacylglycerol [24] were measured on a Cobas-Bio centrifugal analyser (Roche Diagnostica, Basel, Switzerland) using enzymatic kits (Hofmann-La Roche Diagnostica, Basel, Switzerland) and standard control sera. Serum HDL-cholesterol concentrations were measured after precipitation of apoB-containing lipoproteins by polyethylene glycol 6000. The CVs for the individual lipids were all < 5 %. The following modification of the Friedewald equation [25] for molar concentrations was used to calculate LDL cholesterol in mmol/l: LDL cholesterol = total cholesterol – (triacylglycerol/2.18) – HDL cholesterol.

Carotenoids, vitamin E and retinol (only measured in study one)

Plasma carotenoids, vitamin E and retinol were measured once at the end of each phase. After an overnight fast, blood samples were collected using EDTA as an anticoagulant, the plasma separated by low-speed centrifugation and frozen immediately in liquid nitrogen and then stored at –80 °C until analysis. Plasma extractions and HPLC chromatography were performed according to the method of Yang and Lee [26] with minor modifications to this method based on Khachik et al. [27]. Only a small number of samples were processed at a time to minimise exposure to laboratory conditions. The lighting in the laboratory was minimal throughout sample preparation and amber vials were used for the storage of the final extract. The internal standard (α tocopherol acetate) was added and an equal volume of ethanol was then added. Vitamins and carotenoids were extracted with hexane and the extract evaporated to dryness under nitrogen. Extracts were then stored at –20 °C. Mobile phase was used to re-dissolve the samples ready for HPLC analysis. Each volunteer had four plasma samples taken during the course of the intervention study. All samples of a volunteer were extracted in duplicate and analysed in one run on the HPLC to minimise the effect of day to day variation. A standard reference material (National Institute of Standards and Technology product 968b) was initially tested after preparation of the standards (October 1999). All vitamins and carotenoids at the high, medium and low levels fell within the certified ranges. A quality control (QC) plasma was prepared for this study by pooling ~ 20 ml plasma which was mixed thoroughly and aliquots of 500 µl were stored into vials and run with each batch of samples. QC plasma was also stored at –80 °C. A Shimadzu LC 10 HPLC fitted with a refrigerated autosampler and a SPD-M10Avp photodiode array detector with a class LC-10 chromatography workstation was used for analysis of the prepared samples. Isocratic separations of the fat-soluble vitamins and carotenoids were carried out on a Rainin (4.6 mm ID × 250 mm length) C18 (5 µm spherical particles) reverse phase column (Rainin, Woburn, MA, USA). The mobile phase was a mixture of acetonitrile (55 %), methanol (22 %), hexane (11.5 %) and dichloromethane (11.5 %) at a flow rate of 1.0 ml/min. Ammonium acetate (0.01 % w/v) was added to the mobile phase for stabilisation of the carotenoids. Wavelengths of 292 nm (α-tocopherol and α-tocopherol acetate), 325 nm (retinol), 450 nm and 472 nm (carotenoids) were monitored throughout each run. Plasma carotenoid concentrations were standardised for plasma lipid (total cholesterol). Trans α- and β-carotene, lycopene, lutein, retinol, α-tocopherol and α-tocopherol acetate were all obtained from Sigma Chemical Co., St Louis MO. Solvents, hexane, methanol, acetonitrile and dichloromethane were all analytical HPLC grade while the ethanol was 99.5 % HPLC-grade absolute ethanol.

Statistical analysis

Repeated-measures analysis of variance (ANOVA) was calculated with dietary treatment as the within-subject factor and with gender and order as the between subject factors. Age, baseline LDL-cholesterol and BMI were inserted into the model as co-variates. When a significant treatment effect was detected by repeated measures ANOVA, post hoc tests were used to locate differences using a Bonferroni correction. Analyses were performed with SPSS 10.0 for WINDOWS (SPSS Inc, Chicago, USA). For study one, statistical significance was set at $p < 0.01$ in view of the very large number of variables tested except for the contrast of no plant sterols versus milk and margarine combined when $p < 0.05$ after Bonferroni correction was acceptable. For study two, significance was set at $p < 0.05$.

Results

Dietary intakes

In study one, energy intake was apparently 7 % higher in the PSteE-enriched milk phase compared to the PSteE-enriched margarine phase ($p < 0.01$) which was not mirrored by body weight changes suggesting this finding is not representative of the whole dietary period. Body weights were 75.3 ± 10.5, 75.5 ± 10.6, 75.5 ± 10.4 and 74.4 ± 10.6 kg, respectively after the control, PSteE-spread, PSteE-milk or PSteE-milk plus PSteE-spread periods. Total fat and saturated fatty acid intakes were not significantly different between intervention periods. No differences in other dietary variables were noted (Table 2). No changes occurred with time. The diet records from two subjects were incomplete and are therefore not included in the table.

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Table 2 Mean daily nutrient intakes during each treatment period as assessed by weighed food diaries at the end of each intervention period# (study 1)

Nutrient	Control group	PSteE-spread* group	PSteE-milk* group	PSteE-milk plus PSteE-spread** group
Energy (kJ)	8835 ± 1681[1, 2]	8579 ± 1434[1]	9158 ± 1603[2]	8742 ± 1674[1, 2]
Protein (Energy %)	17.4 ± 2.4	17.5 ± 2.0	16.9 ± 2.1	17.1 ± 1.7
Total fat (Energy %)	30.0 ± 4.3	30.0 ± 4.8	30.6 ± 4.8	30.3 ± 4.3
SAFA (Energy %)	11.3 ± 2.4	11.1 ± 2.3	11.6 ± 2.6	11.3 ± 2.0
PUFA (Energy %)	7.8 ± 1.1	8.0 ± 1.2	7.7 ± 1.3	7.9 ± 1.1
MUFA (Energy %)	11.8 ± 2.4	11.8 ± 2.5	12.0 ± 2.3	12.0 ± 2.3
Fibre (g)	24.5 ± 6.3	25.3 ± 6.1	24.5 ± 5.4	25.0 ± 6.2
Alcohol (g)	3.3 ± 4.6	2.7 ± 3.7	3.5 ± 4.5	3.4 ± 4.1
Cholesterol (mg)	247 ± 138	233 ± 104	259 ± 101	231 ± 110

Data are represented as mean ± SD; # represents the mean of 6 days per treatment period; * provided 2 g/d of plant sterols; ** provided 4 g/d of plant sterols; [1, 2] Variables with a common superscript are not significantly different ($P < 0.01$ after Bonferroni correction)

Nutrient intake data in study two are presented in Table 3. There were no significant differences in nutrient intakes between the treatment periods. Body weights and BMI of subjects did not change significantly during the study (data not shown).

For study 1, compliance with the milk drinks was excellent: 100 % compliance was achieved in 24 out of 39 subjects, 14 achieved greater than 95 % compliance and 1 subject was 83 % compliant – this volunteer still achieved a fall in LDL of 0.21 mmol/L. Margarine usage was by phase: 23.0, 24.0, 23.6 and 23.3 g/day and by treatment 23.3, 23.9, 23.6 and 23.0 g (no sterols, margarine, milk, both).

For study 2, compliance was 99.6 %. Nineteen tubs of yoghurt were not consumed out of a possible 5040 tubs. This was due to 4 subjects missing 2 tubs and 11 subjects missing 1 tub each out of a total of 42 tubs per person.

Table 3 Mean daily nutrient intakes during each treatment period as assessed by food frequency questionnaire at the end of each intervention period (study 2)

Nutrient	Control yoghurt group	PStaE-yoghurt* group	PSteE-yoghurt** group
Energy (KJ)	7997 ± 2472	7965 ± 3088	8150 ± 2890
Protein (Energy %)	18.9 ± 2.6	19.0 ± 2.9	19.4 ± 3.1
Total fat (Energy %)	30.8 ± 5.5	30.6 ± 5.7	30.5 ± 6.2
SAFA (Energy %)	11.5 ± 2.9	11.3 ± 2.9	11.2 ± 2.7
PUFA (Energy %)	5.6 ± 2.2	5.5 ± 2.0	5.4 ± 2.1
MUFA (Energy %)	11.0 ± 2.2	11.0 ± 2.3	11.1 ± 2.6
Carbohydrates (E %)	44.9 ± 6.7	45.4 ± 5.6	44.7 ± 7.0
Fibre (g)	24.2 ± 8.5	23.6 ± 9.7	23.7 ± 8.3
Alcohol (g)	12.5 ± 16.9	11.7 ± 16.5	12.9 ± 18.3
Cholesterol (mg)	236 ± 82	242 ± 110	258 ± 129

Data are represented as mean ± SD; * provided 1.7 g/d of plant sterols; ** provided 1.8 g/d of plant sterols; there were no differences in nutrient intakes between intervention periods

Serum lipids and lipoproteins

Study one: In the plant sterol-free period both TC and LDL-cholesterol concentrations (Table 4) were higher than in the PSteE-milk, PSteE-spread and combined PSteE-milk plus PSteE-spread periods ($p < 0.001$ for each with Bonferroni correction). The higher dietary plant sterol intake with PSteE-milk and PSteE-spread combined (4 g/d of plant sterols) did not result in a statistically greater cholesterol reduction than either PSteE-milk or PSteE-spread (2 g/d of plant sterols) alone. The study had sufficient power to detect only a full additive response. The decrease in LDL-cholesterol was 10.1 % with the PSteE-spread (mean, SD 0.49 ± 0.53 mmol/l; 95 % confidence interval (CI) 0.32–0.66), 7.9 % with the PSteE-milk (mean, SD 0.38 ± 0.37 mmol/l; 95 % CI 0.26–0.50) and 11.4 % with the combination of PSteE-milk plus PSteE-spread (mean, SD 0.55 ± 0.46 mmol/l 95 % CI 0.40–0.70). None of the subjects was non-responsive, i. e. experienced no change or an increase in LDL-cholesterol after plant sterol intake in at least one of the three active phases in comparison with the plant sterol-free period. In order to remove some of the variability associated with cholesterol measures, the responses were averaged over all 3 active treatment periods. Sixty-five percent of the subjects showed a decrease in LDL-cholesterol of at least 5 % as averaged over the three plant sterol periods. The correlation between the PSteE-milk response and combined PSteE-milk plus PSteE-spread response was 0.47 ($p = 0.003$), and the correlation between the PSteE-spread response and the combined PSteE-milk plus PSteE-spread response was 0.72 ($p = 0.0001$), but the correlation between the PSteE-milk and PSteE-spread was only 0.3 ($p = 0.06$). This was unrelated to treatment order or compliance. There was also no relationship between baseline LDL-cholesterol, age, BMI, gender or treatment order and response to plant sterol intake. HDL-cholesterol and triacylglycerol con-

Table 4 Mean plasma lipid concentrations after intakes of PSteE-enriched milk or PSteE-enriched spread or the combination of both (study 1)

mmol/l	Baseline	Control group	PSteE-spread group	PSteE-milk group	PSteE-milk plus PSteE-spread group
Total cholesterol	6.83 ± 0.82[1]	6.86 ± 0.93[1]	6.31 ± 0.84[2]	6.48 ± 0.82[2]	6.28 ± 0.80[2]
Triglycerides	1.67 ± 0.71[1]	1.66 ± 0.73[1]	1.61 ± 0.64[1]	1.69 ± 0.71[1]	1.54 ± 0.64[1]
HDL-cholesterol	1.26 ± 0.39[1]	1.27 ± 0.35[1]	1.24 ± 0.39[1]	1.26 ± 0.34[1]	1.30 ± 0.39[1]
LDL-cholesterol	4.83 ± 0.79[1]	4.84 ± 0.82[1]	4.35 ± 0.78[2]	4.46 ± 0.76[2]	4.29 ± 0.74[2]

Values are presented as mean ± SD; [1, 2] variables with a common superscript are not significantly different ($P < 0.01$ after Bonferroni correction)

centrations were not affected by plant sterol intakes (Table 4).

Study two: Both PSteE- and PStaE-enriched yoghurts reduced LDL-cholesterol significantly as compared to the control group by 0.27 ± 0.05 mmol/l (6%) and 0.23 ± 0.05 mmol/l (5%) respectively ($P < 0.001$) (Table 5). The full cholesterol-lowering effect was already noted in the first week with no significant further reduction at weeks 2 and 3 of each intervention (data not shown). There was no significant effect of either plant sterols or stanols on plasma HDL cholesterol or triacylglycerol concentrations.

Plasma carotenoids (study one)

Because of the decrease in LDL-cholesterol, the major carrier of carotenoids and vitamin E, only lipid-adjusted values (carotenoids divided by total cholesterol) are considered. Unadjusted retinol and vitamin D concentrations did not change. No differences were seen in lipid-adjusted lutein, α-tocopherol or lycopene (Table 6). For α-carotene the overall ANOVA was statistically significant ($p = 0.002$), but post hoc analyses revealed no statistical difference after Bonferroni correction despite a 11% decline with the combined PSteE-milk plus PSteE-spread intake in comparison with the plant sterol-free period. The changes in β-carotene were statistically significant ($p < 0.001$). Both the PSteE-milk intake and the PSteE-milk plus PSteE-spread treatment significantly lowered β-carotene by 10% while the intake of PSteE-spread alone did not result in a significant reduction in β-carotene. The adjusted contrast between the plant sterol-free and the combined PSteE-milk plus PSteE-spread period was $p = 0.02$ for the 14% fall before and after adjustment for baseline β-carotene. The change in adjusted β-carotene between the plant sterol-free and plant sterol-enriched periods was correlated with the change in α-carotene in the same periods ($r = 0.54$, $p < 0.001$) but was unrelated to the change in total cholesterol suggesting the interference with carotenoid absorption may not be directly

Table 5 Mean plasma lipid concentrations after intake of PStaE- and PSteE-enriched yoghurt (study 2)

mmol/l	Baseline	Control group	PStaE-yoghurt group	PSteE-yoghurt group
Total cholesterol	6.52 ± 0.87[1]	6.52 ± 0.83[1]	6.29 ± 0.84[2]	6.23 ± 0.79[2]
Triglycerides	1.39 ± 0.72	1.35 ± 0.65	1.29 ± 0.61	1.32 ± 0.64
HDL-cholesterol	1.42 ± 0.43	1.46 ± 0.42	1.49 ± 0.45	1.46 ± 0.44
LDL-cholesterol	4.48 ± 0.75[1]	4.45 ± 0.74[1]	4.22 ± 0.76[2]	4.18 ± 0.71[2]

Data are represented as mean ± SD. The mean represents the mean of 3 weekly measurements; [1, 2] Variables with a common superscript are not significantly different ($P < 0.01$ after Bonferroni correction)

Table 6 Effect of dietary PSteE intake on plasma carotenoids adjusted for total cholesterol (carotenoid/TC μmol/mmol) (study 1)

μmol/mmol	Baseline	Control group	PSteE-spread group	PSteE-milk group	PSteE-milk plus PSteE-spread group
Lutein	0.069 ± 0.029	0.064 ± 0.025[1]	0.068 ± 0.028[1]	0.065 ± 0.028[1]	0.062 ± 0.026[1]
Tocopherol	4.88 ± 0.07	4.89 ± 0.08[1]	5.13 ± 0.09[1]	5.02 ± 0.07[1]	4.87 ± 0.07[1]
Lycopene	0.13 ± 0.07	0.11 ± 0.07[1]	0.12 ± 0.08[1]	0.11 ± 0.07[1]	0.11 ± 0.06[1]
α-Carotene	0.021 ± 0.015	0.018 ± 0.013[1]	0.019 ± 0.014[1]	0.016 ± 0.010[1]	0.016 ± 0.011[1]
β-Carotene	0.076 ± 0.053	0.067 ± 0.042[1]	0.068 ± 0.044[1]	0.060 ± 0.038[2]	0.058 ± 0.037[2]

Data are represented as mean ± SD; [1, 2] Variables with a common superscript are not significantly different ($P < 0.01$ after Bonferroni correction)

related to the interference with cholesterol absorption, although increases in cholesterol synthesis may complicate the interpretation.

Safety data

Values for biochemical (including vitamin D) and haematological parameters, dipstick urinalysis, liver function (γ-GT) as well as adverse event questioning revealed no significant differences between treatment periods (data not shown).

Discussion

In these two studies the cholesterol-lowering effect of PSteE incorporated into low-fat milk and yoghurt was assessed, which makes this one of the few trials that has specifically tested the efficacy of plant sterol-enriched dairy foods in lowering serum cholesterol concentrations. In a single blind, crossover study with 39 moderately hypercholesterolemic subjects, daily intake of low-fat milk providing 2 g of plant sterols (delivered as PSteE) was effective in significantly lowering cholesterol concentrations. In fact, the PSteE were equally effective in lowering LDL-cholesterol regardless of whether they were incorporated into an aqueous matrix such as the milk (8 % lowering of LDL-cholesterol) or in a fat matrix such as the spread (10 % lowering of LDL-cholesterol). No additional benefit in reducing LDL-cholesterol was observed when increasing the daily dose of plant sterols to 4 g/d as the combined PSteE-milk plus PSteE-spread intervention resulted in 11 % lowering of LDL-cholesterol. These findings are in line with data from a recently published meta-analysis demonstrating that intake of 2 g/d of plant sterols results in a 10 % lowering of LDL-cholesterol with higher dietary intakes above 2 g/d adding little extra effect. According to this meta-analysis the maximum cholesterol-lowering effect was estimated at 11.3 % [7].

To our knowledge, the efficacy of plant sterol-enriched low-fat milk in reducing TC and LDL-cholesterol has previously only been reported in an abstract describing a study which tested the effect of free plant sterols from tall oil [28]. In this study, ingestion of 3-times 90 ml of low-fat milk providing 0.9, 1.8 and 3.6 g/d of free sterols lowered LDL-cholesterol by 7.4 %, 8.6 % and 13.2 %, respectively, relative to placebo. Thus, these and our data demonstrate that milk is a suitable food carrier for plant sterol enrichment and that 1.8 to 2 g/d of plant sterols result in a 8–9 % reduction in LDL-cholesterol, which is of similar magnitude as previously described for the same dose of plant sterols incorporated into fat-based spreads.

Our second study, a double blind, crossover trial with 40 subjects also demonstrated the effect of PSteE and PStaE-fortified low-fat yoghurts (1.7–1.8 g/d of plant sterols or stanols) in significantly lowering LDL-cholesterol by 5–6 % as compared to the control yoghurt. The full cholesterol-lowering effect was already reached after 1 week with no further reductions occurring during the second and third week of the intervention. Plant sterols exert their cholesterol-lowering action by inhibiting intestinal cholesterol absorption although the exact underlying mechanisms are not yet fully elucidated [29]. Apparently, intestinal cholesterol absorption is rapidly inhibited after the start of plant sterol ingestion, which explains why the full cholesterol-lowering effect was already seen after one week of PSteE intake. Similar findings have been reported for PStaE as well [11, 30].

Moreover, a 6-day ingestion of 1.8 g/d of non-esterified plant sterols, which were solubilised in low-fat milk, resulted in a significant decrease in cholesterol absorption of 39 % [31], which will consequently result in a decrease in serum cholesterol concentrations. Although these data show that a significant cholesterol-lowering effect can be reached within one week with PSteE or PStaE intake, one should keep in mind that a new metabolic steady state in serum cholesterol concentrations is usually attained within 3–4 weeks.

Regarding low-fat yoghurt, Mensink et al. [11] demonstrated with a PStaE-enriched low-fat yoghurt a reduction of 13.7 % in LDL-cholesterol relative to placebo. This suggests a more pronounced effect as compared to the 5–6 % lowering of LDL-cholesterol with PSteE and PStaE in our study. Apart from differences in the design of the study, e. g. parallel design versus crossover design of our study, there are other important aspects to consider. In the study by Mensink et al. the dose was 3 g/d of plant stanols, which was 1.7-times higher than the dose of 1.7–1.8 g/d of plant stanols or sterols in our study. In addition, three cups of yoghurt (150 ml each) were consumed daily together with meals providing 1 g stanols per serving. In contrast, subjects in our study consumed one serving of yoghurt (150 g) twice per day without further restriction. Whether these differences in amount of servings and time of administration are fully responsible for the different efficacy remains unclear, although is seems plausible that time of administration and intake of plant sterol together with meals may affect efficacy. A previous study [32] has, however, demonstrated that the efficacy of plant stanols in margarine was similar irrespective of the frequency of consumption. Therefore, the difference in the ingested dose of plant sterols versus stanols remains the most likely explanation for the different extent in TC and LDL-cholesterol reduction.

Nevertheless, the cholesterol-lowering effects observed after the 3-week intakes of low-fat milk and yoghurt are generally in line with findings from previously

published studies, which showed that yoghurt, a yoghurt-type drink, or bread enriched with plant sterols or stanols significantly reduced plasma cholesterol concentrations [9–11, 28]. These findings further demonstrated that the cholesterol-lowering effect of plant sterols is not necessarily compromised by an aqueous low-fat food matrix.

Many studies have examined the effects of plant sterols or stanols on fat-soluble vitamins and carotenoids. Concerning β-carotene concentrations (only measured in study 1), there was a borderline significant 11–14% reduction in lipid-adjusted β-carotene levels after the intake of the PSteE-milk (2g plant sterol/d) and the combination of PSteE-milk plus the PSteE-spread (4g plant sterols/d). Lipid-adjusted α-carotene levels were also lowered by 11% but this was not statistically significant. A previous study by Noakes et al. [33] showed that increased consumption of 1 serving of a carotene-rich fruit or vegetable was effective in preventing a similar magnitude of reduction in plasma carotene levels observed after intake of PSteE-rich spreads.

The observed variations in plasma carotenoids are well within observed seasonal and individual variations. Therefore, possible health implications of consuming plant sterol-enriched products in view of carotenoid-lowering is considered to be minimal and adverse health outcomes are not expected [7]. The higher dose of plant sterols of 4 g/d ingested with the combination of PSteE-milk plus PSteE-spread also had no apparent adverse effects on routine safety measures, which is in accord with previous studies with an even higher plant sterol intake of 8.6 g/day [34].

In conclusion, our findings show that low-fat dairy-based food products, like milk and yoghurt enriched with plant sterol esters, are similarly effective in lowering TC and LDL-cholesterol concentrations as fat-based foods like spreads and margarine. Thus, the range of foods that can be enriched with plant sterol esters can be expanded to include low-fat dairy foods such as plant sterol-enriched milk and yoghurt. These foods are perceived as nutritious and healthy and can be easily integrated into a heart healthy diet helping to maintain desirable cholesterol levels or providing an additional dietary option to help lower elevated cholesterol levels.

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