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Abstract

Consumption of plant sterol esters reduces plasma LDL cholesterol concentration by inhibiting intestinal cholesterol absorption. Commercially available plant sterol esters are prepared by esterifying free sterols to fatty acids from edible plant oils such as canola, soybean, and sunflower. To determine the influence of the fatty acid moiety on cholesterol metabolism, plant sterol esters were made with fatty acids from soybean oil (SO), beef tallow (BT), or purified stearic acid (SA) and fed to male hamsters for 4 wk. A control group fed no plant sterol esters was also included. Hamsters fed BT and SA had significantly lower cholesterol absorption and decreased concentrations of plasma non-HDL cholesterol and liver esterified cholesterol, and significantly greater fecal sterol excretion than SO and control hamsters. Cholesterol absorption was lowest in hamsters fed SA (7.5%), whereas it was 72.9% in control hamsters. Cholesterol absorption was correlated with fecal sterol excretion ($r = -0.72$, $P < 0.001$), liver cholesterol concentration ($r = 0.88$, $P < 0.001$), and plasma non-HDL cholesterol concentration ($r = 0.85$, $P < 0.001$). A multiple regression model that included each sterol ester type vs. cholesterol absorption indicated that intake of steryl stearate was the only dietary component that

contributed significantly to the model ($R^2 = -0.75$, $P < 0.001$). Therefore, our results demonstrate that BT and SA are more effective than SO in reducing cholesterol absorption, liver cholesterol, and plasma non-HDL cholesterol concentration, suggesting that cardioprotective benefits can be achieved by consuming stearate-enriched plant sterol esters.

Abbreviations: BT, diets containing plant sterols esterified to fatty acids from beef tallow; SA, diets containing plant sterols esterified to purified stearic acid; SO, diets containing plant sterols esterified to fatty acids from soybean oil.

Introduction

The direct association between plasma LDL cholesterol concentration and risk for coronary heart disease is well established (1). The most recent guidelines of the National Cholesterol Education Program indicate an LDL cholesterol goal of < 2.6 mmol/L (100 mg/dL) for high-risk individuals and < 1.8 mmol/L (70 mg/dL) for very high-risk individuals (2). However, the mean LDL cholesterol level of U.S. adults exceeds 3.1 mmol/L (3), so achieving the target LDL goals generally requires therapeutic intervention. Statin drugs provide effective cholesterol-lowering therapy and are widely prescribed, but they can cause severe adverse effects (4). Lowering plasma LDL cholesterol through nondrug strategies, such as consuming specific food components, would therefore be most desirable.

Consumption of foods enriched with plant sterols (or stanols) in free or esterified form can significantly lower plasma LDL cholesterol concentration. At least 40 clinical studies have been conducted in recent years that indicate a daily dose of 1–3 g of plant sterols significantly reduces plasma LDL cholesterol concentration 10–15% (5,6). It appears that a dose-response relation is continuous up to ~2 g/d (5,7). As a secondary therapy, adding plant sterols to statin medication is more effective than doubling the statin dose (5). Accordingly, the National Cholesterol Education Program recommends 2 g/d of plant sterol or stanol esters as a therapeutic option for reducing plasma LDL cholesterol concentration (8).

The primary cholesterol-lowering mechanism of plant sterols is their ability to reduce intestinal cholesterol absorption (9). Inverse correlations between plant sterol intake and cholesterol absorption have been reported in rabbits (10), hamsters (10,11), and humans (12), and positive correlations between cholesterol absorption and plasma LDL cholesterol concentration have been found in humans (13,14). Consumption of plant sterols also promotes fecal cholesterol excretion in rats (15), hamsters (11), and humans (16,17).

Plant sterols are often esterified to improve their functionality and incorporation into food products. In the 1970s, Mattson and colleagues (18–20) discovered that esterifying plant sterols with long-chain fatty acids increased their solubility in oil 10-fold and that esterification did not impair their ability to inhibit cholesterol absorption. Commercial preparations of plant sterol esters utilize common vegetable oils (e.g., canola, soybean, and sunflower) as the fatty acid source. The extent to which the fatty acid moiety of plant sterol esters influences cholesterol absorption is not known. In this study, plant sterol esters were prepared with variable amounts of saturated and unsaturated fatty acids to test their effects on cholesterol absorption, sterol balance, and plasma cholesterol concentration.

Materials and Methods

Plant sterol ester preparation

Unesterified (free) sterols from soybeans were obtained from Archer Daniels Midland and were composed of ~48% sitosterol, 27% campesterol, and 21% stigmasterol as determined by gas chromatography using a DB-5 capillary column. The free sterols were esterified with fatty acids from refined soybean oil, beef tallow, or purified stearic acid.

Prior to esterification, free fatty acids were prepared from the edible oil triglycerides. Approximately 100 g of oil/fat was added to 360 mL of a 2.5-mol/L KOH solution in ethanol: water (1:1, v:v). The mixture was heated in a boiling water bath for 30 min, then added to 3500 mL of a 3.4-mol/L NaCl solution while stirring. The mixture was poured through a Buchner funnel and washed with ~500 mL of ice-cold deionized water to remove excess KOH and glycerol. The fatty acid salts were spread onto a clean plastic tray and dried in a vacuum oven at 50°C. After dissolving fatty acid salts in 4000 mL deionized water, a molar excess of 6 mol/L HCl was added and stirred for 30 min, forming insoluble fatty acids. The mixture was poured through a Buchner funnel and washed with ~500 mL deionized water to remove excess HCl and KCl. Fatty acids were transferred to a plastic tray and dried in a vacuum oven at 50°C.

The esterification procedure was a modification of a method by Kuksis and Beveridge (21). Free fatty acids were first converted to the acid chlorides by reacting with oxalyl chloride in benzene with dimethyl formamide as a catalyst. The mixture was then concentrated by evaporation under vacuum. Unesterified plant sterols were dissolved in a clean flask containing ethanol-free chloroform and 2 molar equivalents of pyridine. A solution containing a slight molar excess of the fatty acid chloride in ethanol-free chloroform was added dropwise and the mixture was stirred overnight to maximize the esterification reaction. The reaction mixture was diluted with water and the aqueous layer was acidified with HCl to pH 3–4. The separated chloroform layer was dried over sodium sulfate and concentrated under vacuum. The resulting solid was redissolved in hot ethanol; cooling the solution resulted in selective recrystallization of the plant sterol esters. The esters were collected by vacuum filtration, washed with excess cold ethanol, and analyzed for purity by thin-layer chromatography using a solvent system of hexane:diethyl ether:acetic acid (70:30:1, by vol). Plant sterol esters represented > 90% of the product in each preparation, with the remainder consisting mainly of unesterified free sterol.

Water activity, moisture, solubility, specific gravity, and fatty acid distribution were determined for each plant sterol ester preparation (Table 1). Water activity was assessed at 24°C using an Aqualab system (Decagon Devices) and was based on a standard of 6.0 mol/L NaCl in water. Moisture was determined by drying samples overnight at 130°C. The solubility and specific gravity were measured in 4 different oils: canola, olive, soybean, and sunflower. Solubility was determined by adding the esters in small increments and observing the concentration at which a cloudy appearance was maintained when stirred for several hours at room temperature. Specific gravity was performed using a pycnometer at room temperature. Fatty acid composition was determined by gas chromatography of fatty acid methyl esters (22).

Table 1. Characteristics of plant sterol esters[1]

	SO	BT	SA
Water activity	0.60	0.63	0.58
Moisture, *g/100 g*	< 1	< 1	< 1
Solubility in oil, *g/100 g*	5.73	1.26	0.89
Specific gravity	0.98	0.98	0.98
Fatty acid profile, *g/100 g total fatty acids*			
Palmitic acid	11.7	37.5	3.2
Stearic acid	3.4	18.6	96.8
Oleic acid	22.8	28.8	nd[2]
Linoleic acid	59.6	2.1	nd

1. Soybean sterols esterified with fatty acids from SO, BT, or SA.
2. Not detected.

Animals and diets

Thirty-five male F₁B Syrian hamsters (BioBreeders) weighing ~90–100 g were housed individually in polycarbonate cages with sawdust bedding. Upon arrival, the hamsters were randomly divided into groups of 8–9 hamsters and given free access to food and water throughout the 4-wk study. Hamsters were kept in a humidity-controlled room at 25°C, using a 12-h light/dark cycle for the duration of the study. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska.

Hamsters were fed a control diet (devoid of plant sterol esters) or diets containing 50 g/kg plant sterol esters esterified with fatty acids from soybean oil (SO), beef tallow (BT), or purified stearic acid (SA) (Table 2). The AIN-93 mineral and vitamin mixes and beef tallow were purchased from Dyets. The dietary protein source was 90% lean ground beef purchased at a local grocery store and freeze-dried at the Food Processing Center at the University of Nebraska. The freeze-dried beef was pulverized and sifted to provide a homogenous powder. After the ground beef was freeze-dried, its composition of protein, fat, and ash was 64, 32, and 3 g/100 g, respectively. Ground beef was added to the diets at 500 g/kg, thus contributing 160 g fat/kg diet. The fatty acid moiety of plant sterol esters contributed an additional 20 g fat/kg diet; the control diet contained 20 g/kg of BT to mimic the amount of additional fat contributed by the plant sterol esters. Fiber was added to the control diet at 30 g/kg to substitute for the sterol moiety contributed by plant sterol esters. Therefore, all diets contained 180 g fat/kg. The energy distribution for fat, carbohydrate, and protein in each diet was 36, 35, and 29%, respectively. All ingredients were combined and mechanically mixed; the BT was first melted at low temperature before adding to the control diet. Diets were stored at –20°C.

Table 2. Composition of hamster diets containing plant sterols esterified with fatty acids from SO, BT, or purified SA

	Control	SO	BT	SA
	g/kg			
Ground beef[1]	500	500	500	500
Cornstarch	305	305	305	305
Sucrose	50	50	50	50
Plant sterol esters	—	50	50	50
Steryl palmitate	—	5.8	18.8	1.6
Steryl stearate	—	1.7	9.3	48.4
Steryl oleate	—	11.4	14.4	0.0
Steryl linoleate	—	29.8	1.1	0.0
Fiber[2]	80	50	50	50
BT[3]	20	—	—	—
AIN-93 mineral mix[4]	35	35	35	35
AIN-93 vitamin mix[4]	10	10	10	10

1. 90% lean, freeze-dried.
2. Solka-Floc 200.
3. Fatty acid composition (g/100 g): palmitic, 25; stearic, 19; oleic, 42; linoleic, 3.
4. Reeves et al. (23).

Experimental procedures

Body wt and food intake were recorded weekly during the 4-wk study. Cholesterol absorption efficiency was determined during wk 3, and feces were collected during wk 4 to quantify fecal neutral steroid and bile acid excretion. Food was removed on d 28 and the hamsters were killed on d 29 by CO_2 asphyxiation. The abdomen and thorax were opened by incision, and blood was collected by cardiac puncture. Plasma was obtained by centrifuging at 4°C at 2000 × *g*; 30 min. The liver was perfused with saline via the hepatic portal vein to remove residual blood, and the liver and small intestine were excised, weighed, and immediately frozen in liquid N_2.

Cholesterol absorption and fecal neutral steroids were measured as previously described (24). Fecal bile acids were measured enzymatically (Wako Chemicals) following extraction and solubilization in Triton X-100 (25). Liver lipids were determined as previously described (25) using enzymatic reagents for total cholesterol (Roche Diagnostics,), triglycerides (Roche), free cholesterol (Wako), and phospholipids (Wako). Liver esterified cholesterol was calculated as the difference between total and free cholesterol. Plasma total cholesterol was measured enzymatically (Roche); HDL cholesterol was quantified after precipitating apoB-containing lipoproteins (Precipitating Reagent 1335–250; Thermo Electron).

Statistical analyses

Summarized data are expressed as means ± SEM. Treatment differences were determined by ANOVA, followed by Tukey's multiple comparison procedure. Differences were considered significant at the $P < 0.05$ level. Data for liver esterified cholesterol were log-transformed prior to statistical analysis. Associations between 2 variables (e.g., liver weight vs. esteri-

fied cholesterol concentration) were assessed using simple linear regression analysis. Associations between multiple variables (e.g., intake of each plant sterol ester type vs. cholesterol absorption) were assessed by stepwise multiple regression. All data were analyzed using SigmaStat 3.0 (SPSS).

Results

Body wt of all hamsters was 93.9 ± 1.2 g at the beginning of the study and increased to 118.2 ± 1.8 g on d 29. Body wt gain and food intake during the 4-wk study did not differ among treatment groups.

Hamsters fed BT and SA diets had plasma non-HDL cholesterol concentrations that were 64 and 73% lower, respectively, than the control group (Table 3). Plasma HDL cholesterol concentration also was reduced in hamsters fed plant sterol esters, although the magnitude of change was much less than the changes in non-HDL cholesterol. Significant reductions in liver free cholesterol occurred in the BT and SA groups, whereas all hamsters fed plant sterol esters had significantly lower liver esterified cholesterol concentrations than control hamsters. Hamsters fed the BT and SA diets had 96 and 99% reductions in liver esterified cholesterol, respectively. Relative liver weights also were less than in control groups in hamsters fed plant sterol esters (Table 3). Liver weight was directly correlated with liver esterified cholesterol concentration ($r = 0.82$, $P < 0.001$) when all data were plotted, suggesting that changes in liver weight were mainly due to the esterified cholesterol content. Liver triglyceride concentration was highly variable among hamsters and no significant treatment effects were found.

Table 3. Plasma cholesterol, liver weight, and liver lipid concentration in hamsters fed diets containing soybean sterols esterified with fatty acids from SO, BT, or purified SA[1]

	Control	SO	BT	SA
Plasma non-HDL cholesterol, *mmol/L*	2.91 ± 0.21[b]	2.78 ± 0.24[b]	1.03 ± 0.06[a]	0.79 ± 0.07[a]
Plasma HDL cholesterol, *mmol/L*	1.77 ± 0.05[c]	1.28 ± 0.03[b]	1.23 ± 0.04[ab]	1.09 ± 0.05[a]
Liver weight, *g/100 g body wt*	3.58 ± 0.08[d]	3.34 ± 0.05[c]	2.86 ± 0.06[b]	2.51 ± 0.07[a]
Liver free cholesterol, *μmol/g*	5.21 ± 0.42[b]	5.10 ± 0.43[b]	4.12 ± 0.60[a]	4.27 ± 0.33[a]
Liver esterified cholesterol, *μmol/g*	26.04 ± 5.24[c]	13.63 ± 3.13[b]	1.10 ± 0.77[a]	0.21 ± 0.28[a]
Liver triglyceride, *μmol/g*	6.00 ± 2.03	9.99 ± 5.92	7.44 ± 3.18	9.09 ± 4.85

1. Values are means ± SEM, *n* = 9, except for control, *n* = 8. Means in a row without a common letter differ, *P* < 0.05.

Cholesterol absorption was significantly reduced in each group fed plant sterol esters compared with control hamsters, with the SA group having the lowest absorption at 7.5% (Table 4). Fecal neutral sterol excretion was significantly greater in all groups fed plant sterol esters compared with control groups, with intake of BT and SA resulting in a greater rate than SO. Fecal bile acid excretion was significantly lower in the BT and SA groups compared with control hamsters, although bile acid excretion represented < 9% of total sterol excretion in the BT and SA groups. Fecal total sterol excretion was therefore significantly greater in all groups fed plant sterol esters than in control hamsters (data not shown).

In addition, cholesterol absorption was correlated with fecal sterol excretion ($r = -0.72$, $P < 0.001$), liver cholesterol concentration ($r = 0.88$, $P < 0.001$), and plasma non-HDL cholesterol concentration ($r = 0.85$, $P < 0.001$).

Table 4. Cholesterol absorption and fecal sterol excretion in hamsters fed diets containing soybean sterols esterified with fatty acids from SO, BT, or purified SA[1]

	Control	SO	BT	SA
Cholesterol absorption, %	72.9 ± 1.8^d	56.4 ± 2.4^c	29.8 ± 2.5^b	7.5 ± 1.9^a
Fecal neutral sterol excretion, $\mu mol/(d \times 100\ g\ body\ wt)$	3.15 ± 0.41^a	5.82 ± 0.48^b	8.17 ± 0.52^c	8.12 ± 0.56^c
Fecal bile acid excretion, $\mu mol/(d \times 100\ g\ body\ wt)$	1.48 ± 0.17^b	1.17 ± 0.18^{ab}	0.77 ± 0.06^a	0.65 ± 0.12^a

1. Values are means ± SEM, $n = 9$, except for control, $n = 8$. Means in a row without a common letter differ, $P < 0.05$.

Because differences in cholesterol absorption were due to differences in fatty acid composition of plant sterol esters, we compared cholesterol absorption with the intake of plant steryl palmitate, stearate, oleate, and linoleate (calculated from dietary plant sterol ester composition in Table 2 and total daily food intake for each hamster). A multiple regression model that included each sterol ester type vs. cholesterol absorption indicated that intake of steryl stearate was the only dietary component that contributed significantly to the model ($R^2 = -0.75$, $P < 0.001$). The intake of steryl stearate in hamsters fed the SO, BT, and SA diets was 16.5 ± 0.3, 89.7 ± 1.6, and 424.7 ± 8.9 $\mu mol/d$, respectively. Fecal sterol excretion, liver cholesterol, and plasma non-HDL cholesterol are plotted as a function of plant steryl stearate intake in Figure 1.

Discussion

Consumption of plant sterol esters reduces plasma LDL cholesterol concentration by inhibiting intestinal cholesterol absorption (9). Commercially available plant sterol esters are prepared by esterifying free sterols to fatty acids from edible plant oils such as canola, soybean, and sunflower. To determine the influence of the fatty acid moiety on cholesterol metabolism, plant sterol esters were made with fatty acids from SO, BT, or purified SA and fed to male hamsters. The goal was to provide plant sterol esters with markedly different fatty acid compositions (Table 2) while keeping the sterol moiety constant. Our results demonstrate that plant sterol esters enriched with stearic acid (i.e., the BT and SA diets) were significantly more effective than SO in reducing cholesterol absorption, liver cholesterol, and plasma non-HDL cholesterol concentration.
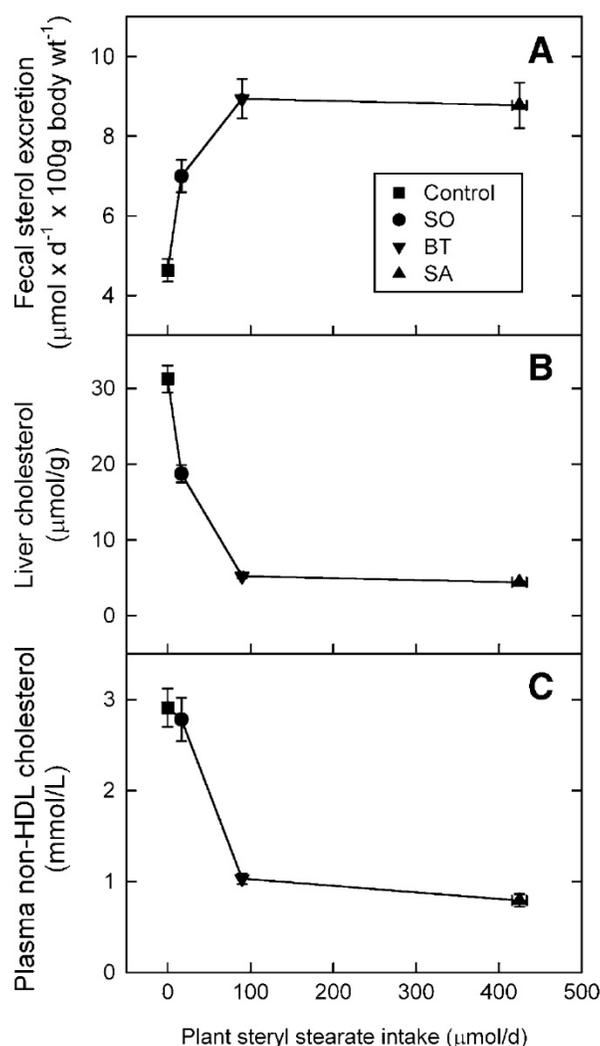
Figure 1. Plant steryl stearate intake and fecal sterol excretion (A), liver cholesterol concentration (B), and non-HDL cholesterol concentration (C) in hamsters fed diets containing soybean sterols esterified with fatty acids from SO, BT, or purified SA. Each point represents the means ± SEM, $n = 9$, except for control, $n = 8$.

The contribution of the fatty acid moiety to the cholesterol-lowering ability of plant sterol esters has been largely overlooked in previous research. Virtually all studies to date have examined plant sterols esterified to fatty acids from common plant oils, as is current practice in the food industry. Three recent animal studies have investigated the metabolic impact of plant sterols esterified to fish oil fatty acids. Male guinea pigs fed plant sterol–fish oil esters had a significantly lower non-HDL cholesterol concentration compared with control groups fed no plant sterols or fish oil (26). Insulin-resistant rats fed plant sterol–fish oil esters had significantly lower serum triglyceride and total cholesterol compared

with rats fed no plant sterols or fish oil (27). Unfortunately, the rat and guinea pig studies lacked either a plant sterol or fish oil control (26,27), so it was not possible to determine whether the results were due to plant sterols per se, fish oil fatty acids per se, or a combined effect of the plant sterol–fish oil esters. Demonty et al. (28) reported that male hamsters fed plant sterol–fish oil esters had a significantly lower non-HDL cholesterol concentration than hamsters fed unesterified plant sterols. Although the results of the hamster study point to fish oil fatty acids as a contributing factor, no other sterol ester preparations were tested, nor did the unesterified plant sterol diet include an equivalent amount of fish oil (28), so the contribution of the fatty acids as part of the sterol ester molecule could not be assessed. A recent human study tested the following dietary preparations: plant sterols esterified to fish oil fatty acids, plant sterols esterified to sunflower oil fatty acids, unesterified sterols plus fish oil, unesterified sterols only, and a control diet without sterols or fish oil (29). The plasma LDL cholesterol concentration did not significantly differ among the treatment groups, suggesting that the fatty acid content (delivered in esterified or unesterified form) did not contribute to the regulation of LDL cholesterol (29). To our knowledge, this is the only published study (29) that directly compares plant sterol ester preparations differing in their fatty acid composition.

Plant sterol esters containing stearic acid were the most potent cholesterol-lowering sterol esters in this study. The cholesterol-lowering or neutral properties of stearic acid, independent of plant sterol intake, are widely recognized, even though the mechanisms of action have not been fully characterized. Stearic acid is a long-chain saturated fatty acid, but it does not raise LDL cholesterol, in contrast to other saturated fatty acids (30). Studies in rats (31–34) and hamsters (24) have shown that stearic acid reduces cholesterol absorption. Dietary stearic acid also reduces liver cholesterol concentration (35,36) and promotes fecal sterol excretion (24,37,38) compared with other saturated fatty acids. Similarly, consumption of plant sterols, independent of dietary fatty acids, produces the same metabolic responses in humans (16,39), rats (15,19), and hamsters (11,40). This study's results (Fig. 1) indicate that reductions in plasma and liver cholesterol, as well as increased fecal sterol excretion, were achieved with relatively modest intakes of steryl stearate (BT diet), suggesting that cardioprotective benefits can be achieved by consuming stearate-enriched plant sterol esters. Because plant sterol esters may be hydrolyzed to some extent in the small intestine (41), the cholesterol-lowering properties observed in this study may be the result of multiple mechanisms involving free stearic acid and free plant sterols. We recently reported that intestinal FHs 74 Int cells incubated with stearic acid, compared with palmitic acid, had significantly reduced gene expression of the cholesterol transporter Niemann-Pick C1 Like 1, suggesting intracellular regulation of cholesterol absorption by stearic acid (42). In a separate study, we reported that plant sterols have the ability to displace cholesterol from micelles in vitro (43), suggesting an intralumenal mechanism that would likely decrease cholesterol solubility in vivo and its ability to be absorbed. The extent to which plant sterols and stearic acid act through independent or synergistic mechanisms will require further study.

Clinical studies have repeatedly shown that plant sterol intakes of 1–3 g/d significantly reduce plasma LDL cholesterol concentration up to 15% and that higher intakes have little added influence (5,6). The majority of human studies included plant sterol esters made

with fatty acids from plant oils. In the current study, hamsters fed the SO diet showed a 5% decrease ($P > 0.05$) in plasma non-HDL cholesterol concentration, which is within the scope of previous human studies. In contrast, hamsters fed BT and SA showed reductions in non-HDL cholesterol of 64 and 73%, respectively. These impressive reductions are much greater than previously reported in humans consuming plant sterols esterified to plant oil fatty acids. It could be argued that plasma cholesterol response to dietary factors is different in hamsters compared with humans, although the modest change observed in the SO group is consistent with humans and does not support the argument. Other studies have noted the similarities in cholesterol metabolism between hamsters and humans (44–47), thus supporting the use of hamsters in modeling the human response to dietary treatment. Nevertheless, a human study that directly tests stearate-enriched plant sterol esters would be helpful in this regard.

In conclusion, BT and SA were significantly more effective than SO in reducing cholesterol absorption, liver cholesterol, and plasma non-HDL cholesterol concentration. The primary cholesterol-lowering feature of BT and SA was enrichment of plant sterol esters with stearic acid. Our findings reinforce the clinical importance of plant sterol esters in the management of hypercholesterolemia, while demonstrating the involvement of the fatty acid moiety. The consumption of stearate-enriched plant sterol esters appears to provide superior benefits to cardiovascular health.

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