Evaluation of the lipid-lowering efficacy of a water dispersible formulation of free sterols versus plant sterol esters in humans consuming a supplemented dairy product

by

Mandana Amir Shaghaghi

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg

Copyright© 2012 Mandana Amir Shaghaghi

ABSTRACT

Reduced bioavailability in some formulations of phytosterols accounts for the variable results observed in LDL- C- lowering efficacy among trials. This study examined the effects of a water-dispersible formulation of free phytosterols (WD-PS) versus phytosterol esters (PS-esters) on plasma lipid and fat soluble vitamins concentrations in hypercholesterolemic individuals. Using a double-blind, randomized, crossover study, 47 hypercholesterolemics were provided for 4 wk: WD-PS-enriched yogurt (2g/d), PS-esters-enriched yogurt (2g/d), or yogurt alone (placebo), in a random order. Each study phase was separated by 4 wk washout intervals. Supplementation of WD-PS or PS-esters similarly decreased serum TC (7.7% and 6.3%, respectively) and LDL-C levels (11.7% and 11.6%, respectively, $p<0.001$). The ratio of TC/ HDL-C decreased for WD-PS (10.6%, $p<0.05$) but not for PS-esters. Moreover, WD-PS reduced serum TG (13.9%, $p<0.05$) as compared to PS-esters (0.6%). The results of the current study confirm the importance of the formulation of phytosterols in their bioavailability and efficacy.

DEDICATIONS

This work is dedicated to people to whom I have been blessed beyond measure by their presence in my life:

My husband, Shahrokh, who is my love, my strength, and my joy. I would like to thank him for his commitment to sharing in this journey of intellectual development.

My mother, Minoo, and my late father, Razi, who have supported me over the years. I would like to thank them for their endless, loving support and their gracious, self-less attitudes.

ACKNOWLEDGMENT

There are several people to whom I would like to express my appreciation for their support and inspiration throughout my Masters' program.

Dr. Peter Jones, thank you for all of your constant support and guidance. With your gracious wisdom and knowledge, you have consistently challenged me to ponder concepts that gently and powerfully furthered my academic and scientific development. Thank you for providing an outstanding climate for my intellectual and professional growth via your willingness to challenge ideas. Thank you for patiently guiding my academic journey as my supervisor and friend.

Dr. Scott Harding, Dr. Mohammad Moghadasian, Dr. David Mymin, Dr. Semone Myrie, Dr. Peter Eck, Dr. Vanu Rampresath and Dr. Natasha Yurkova, thank you for your help to refine my approach to science with your constant supports and tireless advice throughout different stages of my work.

All my lab mates and friends: Yanan Wang, Mohammad Abdullah, Dylan MacKay, Nazanin Vafaei, Kimberly Robinson, Jaclyn Omar, Abraham Girgih, Sandra Castillo, Khatima Khallouf, Haifeng Yang, Andrea Klymasz, and Iptisam Alexanders. Thank you for your friendship and your investment in my development.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1. INTRODUCTION

Background and rationale

Over 40 years ago, cholesterol lowering efficacy of phytosterols (PS) was identified in animals and humans (1). Since the beginning of the last decade, along with rising interest in functional foods, PS have gained much attention and been increasingly incorporated into various food products as functional food ingredients. Numerous in vitro, in vivo, and clinical studies have been done to examine the cholesterol lowering effect, mechanism of action, as well as dose response efficacy of PS. The results of these studies contributed to prove the efficacy of PS by several scientific and regulatory bodies (2-4) which recommend incorporating PS, as a cholesterol lowering nutraceutical component to the diet, to optimize blood lipid concentrations. Indeed, the FDA approved a health claim for PS about 10 years ago. According to the claim, foods containing at least 400 mg per serving of free PS, consumed twice a day with meals, corresponding to a daily total intake of at least 800 mg, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease.

The Adult Treatment Panel of the National Cholesterol Education Program (NCEP ATP III) has drawn attention to the value of PS by recommending the addition of PS (2 g/d) to the diet for primary prevention of cardiovascular disease. Health Canada now permits health claims for coronary heart disease (CHD) risk reduction, based on cholesterol lowering for foods delivering a minimum level equivalent to 0.65 g of free PS per reference amount and per serving of stated size. A combination of PS with diets low in

saturated fat and cholesterol can depress LDL-C levels by 20% or more (5). Thus, plant sterol-enriched products are offering a new avenue in the dietary management of elevated serum cholesterol concentrations which is less expensive compared to pharmacological approaches.

Supplementation of PS at the dosage of 1.8 to 2 g/d have been shown to decrease both total and LDL cholesterol levels by an average of 10% (8 to 15%), yet the results across studies are inconsistent (5). In an attempt to maximize LDL-C-lowering efficacy, considering the factors that influence the heterogeneity of response of individuals to PS is essential. One of the most obvious factors is the proper solubility and bioavailability of every formulation of PS which influence the ability of PS to have an effect upon lipid profiles. Therefore, in view of the important influence of the formulation of PS on their cholesterol lowering efficacy, it may be preferred that every new formulation of PS be examined on their efficacy.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Introduction

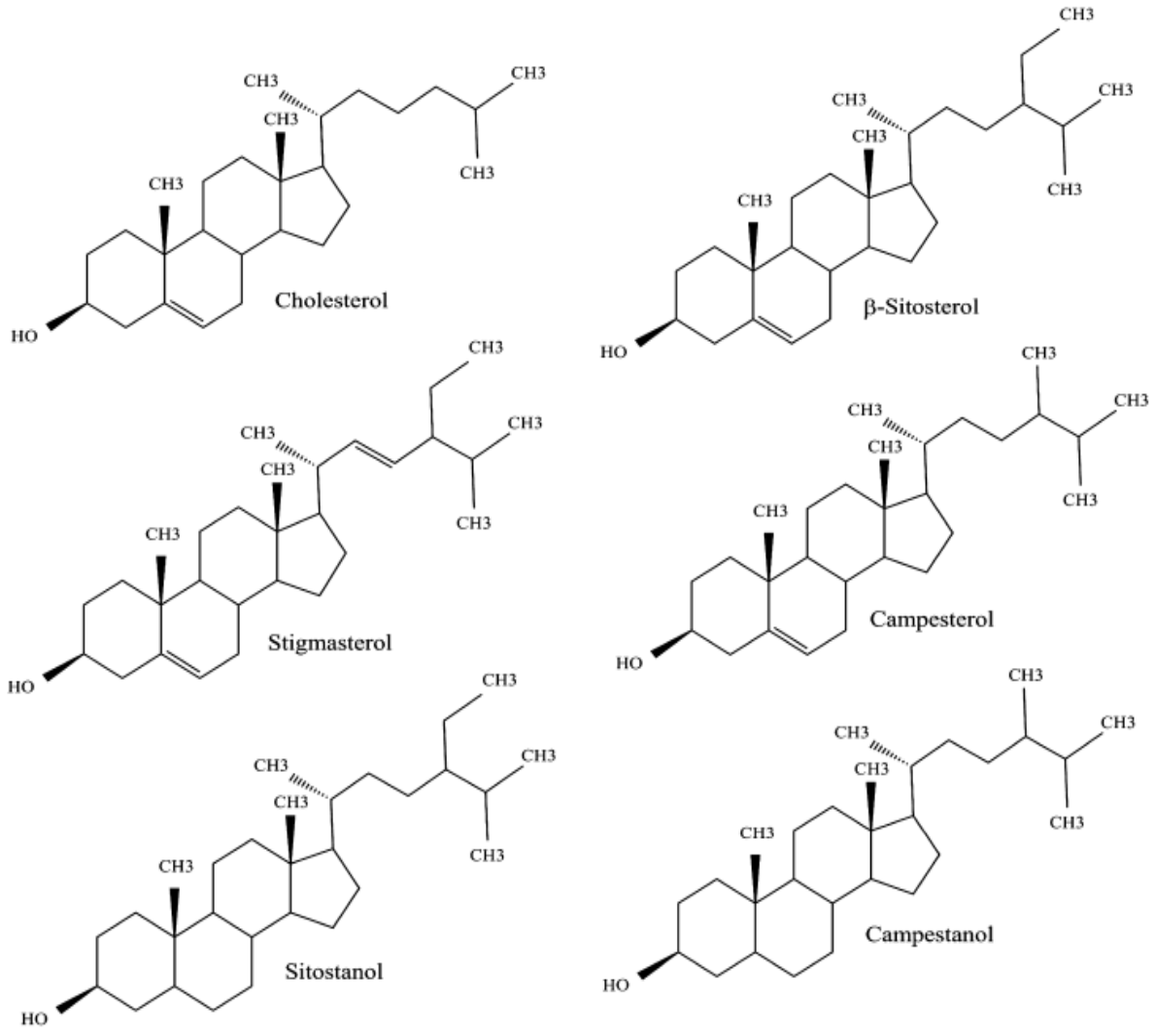
The following review presents the current knowledge surrounding the cholesterol lowering efficacy, mechanisms of action, and factors affecting bioavailability and efficacy of phytosterols for the management of dyslipidemia.

2.2 Plant sterol chemical structure and availability in foods

Phytosterols, encompassing plant sterols and stanols, represent a cluster of plant-derived sterols which are common and share important attributes. Phytosterols are structurally similar to cholesterol, but differ in the side chain attached to the steroid ring, where they have a methyl or ethyl group on the C24 position. Plant stanols are saturated forms of plant sterols which are less abundant in nature than the unsaturated forms of sterols. More than 200 different types of PS have been reported, including β-sitosterol, campesterol, and stigmasterol which are the principal molecular forms of plant sterols and which contain more than 95% of total PS in the human diet (6, 7). In many foods, β-sitosterol is the most abundant form in the human diet followed by campesterol which constitutes approximately 60% and 35%, respectively, of the total dietary PS (8). Major plant sterols and stanols are identified in Figure 1.

In general, similar to function of cholesterol in vertebrate animals, PS serve to stabilize plant cell membranes, whereas, due to different structural properties of individual PS, they have different effects on membrane stability (9).

Figure1. Chemical structure of cholesterol and common plant sterols and stanols



Dietary intake of PS in a western diet ranges from 150-400 mg /d, while for vegetarians it could be as high as 1 g/d (1, 6). All vegetable foods contain appreciable quantities of PS. The most concentrated source of phytosterols is vegetable oil and smaller unit amounts are found in nuts, breads, and whole vegetables.

2.3 Effective dose of plant sterols for cholesterol lowering

Extensive data confirm that consumption of foods enriched with PS significantly reduce TC and LDL-C concentrations without affecting levels of high-density lipoprotein cholesterol (HDL-C) or triglycerides (TG) (10).

In human studies, several researchers have reported that a dose of 1.6–2.4 g/d of PS mainly in the form of PS-ester, lowers TC and LDL-C concentrations equally by 8–15% (11, 12). The dose response relationship between consumption of PS and the efficacy in lowering LDL-C has been established in individual studies (13-15) as well as several meta-analyses (5, 16, 17, 18).

In the first substantial meta-analysis of 18 published clinical trials, Law et al (16) suggested that regular consumption of 2 g/d of PS, added to an average daily portion of margarine and compared with margarine alone, lowers LDL–C concentrations by at least 0.50 mmol/L for individuals aged 50-59 yr and 0.39 mmol/L for individuals aged 40-49 yr. At higher doses, no further reduction in LDL cholesterol was apparent (16). Furthermore, to predict the effect of a given PS dose, Katan et al (5) conducted a systematic meta-analysis of 41 trials which showed a nonlinear dose-response relationship between the daily dose of PS and their cholesterol-lowering efficacy. In the trials assessed, the percentage reduction in LDL-C as a function of dose tapered off at intakes of about 2 g/day or more maximum effect was estimated at 11.3%, while there was little additional effect at doses higher than 2.5 g/d. In a meta-analysis by AbuMweis et al (17), there was evidence of a dose-response effect. The minimum (-0.25 mmol/L;

95% CI: -0.32, -0.18) and the maximum (-0.42 mmol/L; 95% CI: -0.46, -0.39) reduction in LDL-C levels were achieved by the intake of more than 1.5 g/d (study n=8) and less than 2.5 g/d (study n=13) of PS, respectively. Finally, in the most recent meta-analysis of eighty-four trials, Demonty et al (18) established a continuous dose-response relationship that would allow predicting the LDL-C lowering efficacy of different PS doses. On average, PS consumption lowered LDL-C levels by 0.34 mmol/L (95% CI: -0.36, -0.31), which corresponded to a relative decrease of 8.8% (95% CI: -9.4,-8.3) for a mean daily intake of about 2 g PS. The LDL lowering action of PS exhibited a plateau at intakes of 3 g/d.

2.4 Mechanisms by which phytosterols decrease plasma lipid levels

In humans, cholesterol absorption occurs in the small intestine where both dietary cholesterol (300 mg/d) and biliary cholesterol (1000mg/d) are available for uptake from the intestinal lumen (19). Three major segments are involved in cholesterol absorption. The first step takes place intraluminally, where unesterified cholesterol dissolves into the mixed micelle to facilitate its movement up to the brush border membrane of the enterocyte (20). The second phase, which involves the transport of cholesterol across the brush border membrane, occurs by the Niemann-Pick C1 Like-1 (NPC1L1) transport protein, while the third phase, within the enterocyte, involves the esterification of cholesterol and its incorporation into chylomicrons for uptake into circulation (20).

Compared to cholesterol which can be absorbed up to 60%, PS are absorbed only in trace amounts, roughly up to 2% (21). Yet, PS inhibit the absorption of intestinal cholesterol

and hence reduce circulating cholesterol concentrations. The exact mechanism by which PS interfere with cholesterol absorption remains to be clarified, but based on cholesterol's absorption stages, three proposed mechanisms are suggested (21, 22).

2.4.1 Competing with cholesterol for incorporation into mixed micelles

One of the proposed mechanisms is based on the fact that cholesterol must enter bile-salt and phospholipid-containing 'mixed micelles' in order to pass through intestinal cells and be absorbed into the bloodstream.

Under the same mechanism as cholesterol absorption, PS must solubilize into intestinal bile salt micelles to be absorbed from lumen (6). Due to the limited capacity of the mixed micelle to embody lipophilic molecules, the competitive solubilization between PS and intestinal cholesterol results in micellar displacement of cholesterol by PS and less uptake of both dietary and endogenously-produced biliary cholesterol into the enterocyte (9, 23). The process of micellar solubilisation, as a major factor affecting the absorption rate, has been studied in vitro and in vivo (21, 24, 25). Ikeda et al (26) designed a series of in vitro studies to compare the micellar solubility of PS, sitosterol and fucosterol, alone and in equimolar binary mixtures to 2.0 mM. Results revealed that cholesterol has the highest solubility followed by fucosterol and sitosterol. However, in binary mixtures, cholesterol solubility was decreased by sitosterol and to a lesser extent by fucosterol relative to its solubility alone (26). These findings are consistent with a report from another in vitro study (27) which demonstrated the importance of the side chain substitution at carbon 24 to sterol solubilization in taurocholate micelles. Results of the study showed that

sitosterol, with less hydrophilic property than cholesterol, has a lower capacity but higher affinity for binding to cholic acid micelles and is predicted to displace cholesterol with a favorable free energy change. Results from another in vitro study (28) revealed that the rate of sitosterol movement from the micellar phase to triolein was 3.5-fold less than cholesterol and this was consistent with the suggested differences in their micellar affinity. A more recent in vitro study (25) showed that free sitosterol and sitostanol reduce the concentration of cholesterol in dietary mixed micelles via a dynamic competition mechanism and hence, decrease its transport towards the intestinal brush border membrane. The competition mechanism between PS and cholesterol to dissolve into micelles results in decreasing the rate of cholesterol absorption which consequently reduces blood cholesterol concentrations.

In general, the displacement of cholesterol from micelles by plant sterols and the greater displacement with sitosterol and campesterol relative to other PS measured in vitro, are consistent with the observed inhibitory effects on cholesterol absorption in vivo (24, 26). The ability of PS to suppress micellar solubilization of cholesterol is one of the essential factors to decrease intestinal cholesterol absorption and therefore reducing circulating levels of cholesterol, while partially increasing endogenous cholesterol synthesis (29). It should be noted that PS have more hydrophobicity and higher affinity for micelles than cholesterol, and thus their absorption is very poor (7). Indeed, campesterol with highest absorption levels among PS, 3 times higher than β-sitosterol and stigmasterol (9.4-14.8%, 3.1-4.5%, and ≤4%, respectively), still has a much lower absorption rate than cholesterol (30%-60%) (6).

2.4.2. Modulating the action of key cholesterol intestinal transporters

Cellular cholesterol concentration within the enterocyte is regulated by a number of processes including uptake of cholesterol from the lumen by Niemann-Pick C1-Like1 (NPC1L1) protein, and secretion of cholesterol back into the intestinal lumen by adenosine triphosphate-binding cassette G5 (ABCG5) and G8 (ABCG8) transporters, located on the brush border of intestinal cell (7). PS may modulate the action of these key intestinal shuttle transporters involved in cholesterol absorption (NPC1L1, ABCG5, and ABCG8 transporters). Recent evidence suggest that NPC1L1 expression in the small intestine and in the brush border membrane of the enterocyte, is critical for absorption of both cholesterol and PS (19, 30). Findings by Altman et al (19) revealed that NPC1L1-deficient mice exhibit a substantial reduction in absorbed cholesterol which was unaffected by dietary supplementation of bile acids (19). Moreover, Davis et al (30) found that NPC1L1 null mice had not only substantially reduced intestinal uptake of cholesterol but also decreased absorption of sitosterol and thus dramatically reduced plasma PS levels. Interestingly, although, the NPC1L1 null mice had hypercholesterolemia, they were completely resistant to diet-induced hypercholesterolemia (19, 30). Furthermore, plasma lipoprotein and hepatic cholesterol profiles in NPC1L1 null mice were similar to those of wild type mice treated with ezetimibe, the cholesterol absorption inhibitor drug (19, 31). The finding that deletion of the gene for NPC1L1 in mice results in the prevention of PS and cholesterol absorption, may suggest that NPC1L1 protein acts as a common pathway for uptake of PS and cholesterol into the entrocyte which results in reducing the uptake of the latter (7, 20). On

the other hand, ABC transporters, including ABCG5 and ABCG8, are the group of membrane proteins responsible for the transport of cholesterol and PS back into the intestinal lumen (32, 33). Evidence suggests that in sitosterolemia patients, a rare autosomal recessive disorder, plasma and tissue levels of PS are increased 30-200 fold as a result of mutations in these transporters (34). In support of these findings, Yu et al (35) have shown that in the ABCG5 and ABCG8 knockout mice the mean plasma PS concentrations increased more than 30-fold as a result of lack these transporters. In another study involving inbred mice, Duan et al (36) found that the jejunal and ileal ABCG 5 and ABCG8 play a main regulatory role as sterol efflux transporters in response to high dietary cholesterol and sitostanol. Moreover, results of this study demonstrated that the absorption efficacy of cholesterol and sitostanol is mostly determined by the net results of a complex series between influx, through NPC1L1, and efflux, by ABCG5 and ABCG8, of intraluminal cholesterol and sitostanol molecules crossing the apical membrane of the enterocyte (36).

2.4.3 Preventing esterification of free cholesterol

Phytosterols are also thought to prevent esterification of free cholesterol into cholesterol esters and thus preventing its assembly into chylomicrons. Upon entry into the enterocyte, cholesterol is esterified by the enzyme acyl-CoA, cholesterol acyltransferase (ACAT) and then incorporated into chylomicrons for subsequent secretion into the lymphatic system which ultimately reach the liver (7). ACAT has a critical role in cholesterol absorption, as only esterified sterol compounds are incorporated into chylomicrons (37). PS have a low affinity for ACAT, and hence are relatively poorly

esterified by this enzyme, resulting in less passage to circulation than cholesterol (6). Nevertheless, it has been suggested that the intracellular esterification of PS could suppress the activity of ACAT due to higher concentration of free cholesterol, and thus its assembly into chylomicrons and taken up into the circulation (6, 38, 39).

2.5 Plant sterol formulation and cholesterol lowering efficacy

Attempts to measure biological effects of PS in clinical studies have been impeded by limited solubility of PS in both water and fat (1).

Esterification of PS with long-chain fatty acids increases fat solubility by 10-fold and allows great solubility in the oil phase of the fatty foods, providing optimal dispersion within fat-containing micelles (40). Considering the palatability and ready solubility in fatty- based foods, such as margarine, PS-esters have been studied more and shown to have optimal success in LDL-C-lowering efficacy, however, beneficial effects of PS preparations were also demonstrated in several studies when various methods were used to increase bioavailability of free PS (46-48). Free PS can also be dispersed in water after emulsification with lecithin or other emulsifiers and considerably reduce cholesterol absorption and circulating LDL-C when added to non-fat foods (49, 50).

PS, in free or esterified forms, are added to foods for their properties to reduce absorption of cholesterol and thereby lower blood cholesterol levels. It is now generally accepted that free-PS and PS-esters have the same cholesterol lowering efficacy (41), however, in some results across studies such comparisons have been inconsistent which

is likely due in large part to study design, food matrix, and formulation of PS (5, 42, 43). The fact that incorporating PS into the same food matrix did not result in an identical magnitude in LDL-C reduction in different trials (29), shows that LDL-C lowering effect of PS is not based only on their food formatting, but also on their availability for absorption into mixed bile salt micelles. Indeed, reduced bioavailability in some formulation of PS, which are relatively insoluble substances, appears to account in part for the variable results observed in these LDL-C lowering clinical trials (44, 45). The experiments that showed the largest effects of PS used formulations of PS which have high bioactivity and thus LDL-C lowering efficacy.

Although recent analysis demonstrated that free PS and PS-ester incorporated into foods have similar cholesterol lowering action (5, 18, 41), some researchers still question the equivalency of physiological efficacy. Similar to cholesterol, PS-esters must be hydrolyzed, possibly with cholesterol esterase, to free PS which is then solubilized into mixed micelles in the intestinal lumen (52). The mechanism of hydrolyzing PS-esters to free PS in the intestine has been proposed as a rate-limiting process for cholesterol lowering effect of PS-esters which may prove that free PS are most likely physiologically active forms (9). Makoto et al (52), showed that serum cholesterol concentrations, as well as fecal steroid excretion, were significantly higher in rats fed free PS compared to those fed the control diet or PS-ester diets. Moreover, deposition of cholesterol in the liver was significantly lower in rats fed free PS than PS-esters. Makoto et al (52) suggested that hydrolysis of PS-esters in intestinal lumen can be a rate-limiting process which impair a sufficient inhibition of cholesterol absorption in rats. However, another study examined

in the effect of PS formulation on cholesterol lowering efficacy in hamsters, which supported the comparable efficacy of free PS and PS-esters and showed that esterification of PS with fatty acid do not impair the ability of free PS to inhibit cholesterol absorption (53) . A similar observation was also reported in humans, however, direct comparison to clarify differential hypocholesterolemic effect of free PS and PS-esters is scarce (5, 54, 55). Nissinen et al (54) infused PS-esters and free PS in the duodenum of healthy humans and showed that PS-esters can be quickly hydrolyzed, 40% during a 60 cm-intestinal passage, while free PS experienced partial esterification (30%) and sedimentation during intestinal passage (54). These two routes may explain the reason why, in humans, the inhibitory effect on cholesterol absorption is similar for free PS and PS-esters. In another study, Richelle et al (55) showed that the inhibitory effect on cholesterol absorption was comparable between free PS and PS-esters, when normocholesterolemic men consumed a PS-enriched low fat milk beverage for 1 week (55).

2.6 Effect of food matrix on cholesterol lowering efficacy of plant sterols

The plasma cholesterol-lowering efficacy of PS varies according to the composition and the food matrix in which they are provided (56). To date, the impact of the food format on the LDL-C lowering efficacy of PS has been evaluated. Extensive evidence for the cholesterol-lowering efficacy of PS, incorporated in a variety of fat based food products, such as margarine and spreads has become available. Moreover, several studies, assessing the cholesterol-lowering efficacy of dairy products containing plant sterols, suggest that yogurt-based sterols are effective in reducing cholesterol levels in primary moderate

hypercholesterolemia (18, 57). Clifton et al (58) compared efficacy of LDL-lowering following PS supplementation in different food carriers and showed that in 58 hypercholesterolemic men and women, PS esters-enriched low-fat milk reduced LDL-C (15.9%) more effectively than PS-enriched yogurt, bread, and cereal (8.6%, 6.5%, and 5.4%, respectively). The results were similar to the cholesterol-reductions observed by Noakes et al (11) where PS ester were incorporated into fat free/ low fat dairy products. Overall, the above evidence suggests that dairy products are appropriate foods by which PS are delivered and, therefore, effectively exert lipid lowering effects.

2.7 Plasma plant sterols in response to plant sterols feeding

Plasma plant sterols cannot be synthesized endogenously and are thus entirely derived from diet. Consumption of a Western diet results in circulating plant sterol with the major concentration of campesterol up to 0.009 mmol/L following by sitosterol concentration for up to 0.006 mmol/L. These concentrations along with the concentrations of all other PS including stigmasterol and brassicasterol, contribute to less than 1% of total plasma sterols which comprised mainly of cholesterol by concentrations of 5.17mmol/L (59).

In numerous well-controlled human intervention trials, consumption of plant sterol–enriched products increased serum plant sterol concentrations. Although the results of various published clinical data are comparable, some differences also exist. Results from a meta-analysis, including 41 trials (5), indicated that intakes of 2 g/d of plant sterols approximately doubles plasma sitosterol and campesterol levels in hyperlipidemic, but otherwise healthy individuals. It also increases plasma stanols levels, while their

concentrations are always a factor of 10 to 100 fold lower than sterols (5). Also, Clifton (58) observed some increases in plasma β-sitosterol and campesterol levels following consumption of PS in different matrixes including fat free milk, low fat milk, and bread. In a study using PS-enriched oil products, Chan et al (60) showed a significant increase in plasma campesterol, stigmasterol and β-sitosterol concentrations compared with the controlled products. Another study used the combination of fat free/low fat fermented milk with 1.6 g/d of PS and showed an increase in plasma β-sitosterol concentrations of 35% compared with a fermented milk control group (61).

Moreover, in an ongoing free-living Dutch cohort study over a 5-y follow-up period (1999-2003), Fransen et al (62) analyzed baseline and follow-up data on serum plant sterols, plant stanols and cholesterol concentrations for 80 users of plant sterols or plant stanols–enriched margarine and 81 matched nonusers. In this study, cholesterol-standardized serum sitosterol concentrations increased by 22% with long-term plant sterols consumption and decreased by 18% with plant stanols consumption, whereas, standardized campesterol concentrations increased by 103% with consumption of sterol–enriched margarine and decreased by 11% with plant stanols margarine use. Finally, in the most recent clinical literature, Derdemezis et al (63) indicated that the regular intake of 2 g/d of dietary plant sterols results in an increase in plasma concentrations of sitosterol and campesterol by 20% to 100% and 40% to 100% respectively, which in absolute terms, is related to an increase of approximately 0.02 mmol/L in the sum of both plant sterols. They concluded that even after the intake of plant sterols–enriched foods, plasma plant sterol concentrations usually contribute to less than 1% of all plasma sterols

including cholesterol and it seems unlikely that the small absolute increase of plant sterol plasma levels outweighs the well established benefits from TC and LDL-C lowering.

CHAPTER 3. STUDY OBJECTIVES

The objectives of the present study are:

1. To assess the effect of a new formulation of water dispersible PS compared to plant sterol esters, on plasma lipids concentrations, in moderately hyperlipidemic individuals.

2. To determine the effect of a new formulation of water dispersible PS, compared to plant sterol esters, on plasma fat soluble vitamins concentrations in moderately hyperlipidemic individuals.

3. To evaluate the effect of a new formulation of water dispersible PS compared to plant sterol esters, on plasma plant sterol concentrations and cholesterol synthesis markers levels in moderately hyperlipidemic individuals.

4. To estimate the effect of a new formulation of water dispersible PS, compared to plant sterol esters, on plasma markers of hepatic function and inflammation (liver enzymes) in moderately hyperlipidemic individuals.

CHAPTER 4. MANUSCRIPT

Water dispersible plant sterol formulation shows improved lipid lowering efficacy compared to plant sterol esters

Mandana Amir Shaghaghi, Scott V. Harding, and Peter J.H. Jones

This manuscript has been submitted to: Journal of Lipid Research

Funding for the study was provided by: Naturalis S.A., Inc, Chile

3.1 Abstract

Despite repeated demonstration of cholesterol-lowering efficacy of phytosterols (PS), issues surrounding reduced PS bioavailability of some dietary formulations remain to be elucidated. The objective of this study was to determine the efficacy of a water dispersible formulation of a free plant sterol (WD-PS) versus plant sterol esters (PS-esters). Forty-seven hypercholesterolemic subjects completed a free living, double-blind, randomized, crossover study and were provided with either a single-dose daily regimen of PS-enriched yogurt (2g/d of PS from WD-PS or PS-esters) or only yogurt (placebo) for 4 wk. Yogurts enriched with WD-PS or PS-esters induced similar decreases in serum total (7.7% and 6.3%, respectively) and LDL cholesterol levels (11.7% and 11.6%, respectively), as percentage relative to the control ($p<0.001$; all). However, ratios of total to HDL cholesterol and non-HDL to HDL cholesterol decreased ($p<0.05$) with WD-PS (10.6% and 15.2%, respectively) but not with PS-esters (7.0% and 10.8%, respectively) compared with control. Over the treatment period, consumption of WD-PS significantly reduced serum TG levels (13.9%) compared to consumption of PS-esters (0.6%). Both WD-PS and PS-esters contributed effectively to LDL cholesterol lowering, however, the formulation of WD-PS appeared to yield additional lipid lowering effects on preventing cardiovascular diseases by improving serum TG and the ratio of TC to HDL-C.

Key words:

Water dispersible plant sterols, Esterified plant sterols, Cholesterol absorption

3.2 Introduction

According to World Health Organization, cardiovascular diseases (CVD) are the leading causes of death globally (1). Lowering of LDL-C is central in the prevention of CVD and can be achieved through dietary modification and therapy (2). Hence, due to the established cholesterol-lowering effect of PS, several regulatory bodies recommend intake of 2 g/day of PS as a component of a modified diet, to optimize blood lipid levels (2-4). The major mechanism of action responsible for the cholesterol-lowering property of PS is the inhibition of intestinal cholesterol absorption (5). The competitive solubilisation between cholesterol and PS in bile salt micelles purportedly decreases intestinal cholesterol absorption and thus reduces circulating levels of cholesterol, while partially increasing endogenous cholesterol biosynthesis (6). However, owing to the chemical structure of PS and their poor solubility and bioavailability, these compounds must be properly formulated to achieve optimal health benefits. Indeed, PS have crystalline properties, a high melting point, and low solubility in water and fats which complicate their incorporation into food matrices and limit their practical applications (7, 8). Therefore, creating a more soluble PS type product may increase solubility and bio-efficacy.

In principle, different approaches may fabricate delivery systems for PS, but these can be broadly categorized as esterification or emulsification depending on the underlying approach. Traditionally, the most common process is to convert PS to their esterified forms, with vegetable oil fatty acids, for subsequent integration into fat-based foods such as margarine and spreads (8). Furthermore, research in the nutraceutical industry has

shown that the bioactivity of PS can be greatly enhanced by incorporating them within various emulsion-based delivery systems. For instance, formulating free PS with lecithin considerably reduces cholesterol absorption and circulating LDL-C, while less effect was seen with PS in crystalline forms (9, 10). Moreover, Lin et al (11) indicated that natural phytosterol glycosides, purified from soy lecithin, reduced cholesterol absorption by 37.6%, compared to the 30.6% reduction observed simultaneously with PS-esters.

Ultimately, in a meta-analysis of eighty-four trials, Demonty et al (12) confirmed that the dose-dependent LDL-C–lowering efficacy of PS had no noticeable relationship with various treatment characteristics, including fat-based vs. non fat-based formats and/or free-PS vs. PS-esters forms. Indeed, esters of PS similar to those of the free forms have been shown to induce a similar LDL-C–lowering effect when provided at the same free sterol equivalent dose (13-15), however, for some results across studies such comparisons have failed. Decreased bioavailability of free PS, owing to the difficulty of formulating, solubilizing, and delivering these relatively insoluble substances is one of the main causes for the inconsistency among the results of these studies (7, 16). Therefore, based on the critical importance of the form of PS in its bioactivity and efficacy, new formulations should be assessed for value if they differ greatly from previously tested forms.

The objective of the present study was therefore to examine the effects of a new formulation of a water dispersible PS (WD-PS) preparation, compared with a conventional PS-esters form, on serum lipids and fat soluble vitamins concentrations. In

addition, the safety parameters, defined as reported adverse events and/or undesirable changes in clinical chemistry parameters including liver enzymes, was examined during 4 wk consumption of the new formulation of WD-PS. Our primary null hypothesis was that the effect of the new preparation of WD-PS on blood lipid levels would be the same as traditional forms of PS-esters. Thus, we expected plant sterols to have a similar effect on plasma total and LDL cholesterol levels when they are provided either as WD-PS or PS-esters.

3.3 Materials and methods

3.3.1 Treatment preparation

Plain yogurt (4% MF, Dairyland, Saputo, Canada) was used as a food carrier for both PS (WD-PS and PS-ester) and also as the control. PS-esters were esterified with rapeseed fatty acids. The WD-PS with the targeted composition and particle size, using the patent application WO 2010/095067, was prepared and provided by Naturalis S.A., (Santiago, Chile). In brief, WD-PS is a stable, non-decanting, readily-dispersible phytosterol dispersion that does not require high shear mixing or homogenization to be suitably formulated into food products and was incorporated into the yogurt mix through gentle agitation (17).

The composition of WD-PS and PS-esters PS is shown in Table 1. PS-esters contained more campesterol and stigmasterol compared with WD-PS. WD-PS and PS-esters were suspended and flavored in the metabolic kitchen of the Richardson Centre for Functional Foods and Nutraceuticals. No organoleptic differences were detected between the PS-

enriched (WD-PS and PS-esters) and the placebo yogurts. In addition, the suspension was stable throughout the refrigerated shelf-life of the finished yogurt for up to 4 wk.

3.3.2 Study population

Hypercholesterolemic individuals were recruited by advertisements, placed in local Winnipeg newspapers, via educational sessions, and on the Richardson Centre for Functional Foods (RCFFN) website. Every subject approved and signed an informed consent form and this study was approved by the Biomedical Research Ethics Board at the University of Manitoba.

Potential study subjects were initially screened by questionnaire regarding personal health information, medical conditions, and disease history. If subjects were determined to be potentially eligible they underwent blood screening at the first visit where a 10ml fasting blood sample was taken to test for general lipid profiles including TC, HDL-C, LDL-C, and TG levels. Inclusion criteria included baseline LDL-C above 2.8 mmol/L, TG below 4.5 mmol/L, a body mass index (BMI) between 20 and 30 kg/m^2, and age 19-75 yr. Subjects were excluded if they took statins, nicotinic acid, or fibrates. Subjects who were diagnosed to have diabetes mellitus, sitosterolemia, heart disease, liver disease, kidney disease, or who had recently undergone major surgeries were also excluded from the study.

3.3.3 Experimental protocol

The study was a free-living, randomized, crossover trial consisting of three 29 d treatment phases each separated by 4 wk washout intervals. Subjects were assigned to receive the treatments, WD-PS-enriched yogurt, PS-esters-enriched yogurt, or yogurt without PS (placebo), in a random order. During each treatment period, subjects were instructed to consume their normal diet and consume their supper meal in conjunction with the treatment under supervision, on a daily basis to monitor compliance.

3.3.4 Blood sampling and analysis

Twelve-hour fasting blood samples were collected on days 1, 2, 28 and 29 of each of the 3 phases of the trial. Blood samples obtained on days 1 and 2 were used to measure baseline values for different study measurements, whereas blood samples obtained on the last 2 days were used to measure final values. Blood samples were collected and centrifuged for 20 min at 3000 rpm. The separated aliquots were frozen at -80^{O}C until analysis. Plasma TC, TG, HDL-C, and liver enzymes levels were analyzed using a VITROS 350 chemistry autoanalyser (Ortho-Clinical Diagnostics, Markham, ON, Canada). Plasma LDL-C concentrations were calculated using the Friedewald equation (18). Plasma CRP was analyzed using an Adiva 1800 clinical chemistry system (Siemens Healthcare Diagnostic, ON, Canada).

3.3.5 Plasma cholesterol precursor and plant sterol analyses

Plant sterols and precursor sterols concentrations in serum were measured using capillary gas–liquid chromatography GC (Bruker 430; Billerica, MA, USA) based on previously

established methods (19). Briefly, 5α-cholestane as an internal standard (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) was added to each plasma sample (100µg/0.5 mL plasma). Samples were saponified with 0.5 M methanol-KOH for 2 h at 100°C, and the unsaponifiable portion extracted with petroleum ether. The extracted non-saponifiable materials were used to determine PS concentration levels. Samples were derivatized with 500 µl TMS reagent (pyridine–hexamethyldisilazane–trimethylchlorosilane; 9:3:1). After preparation samples were injected into the GC equipped with a flame ionization detector and an auto-injector system. Separation was achieved on a 30 m capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 µm (SAC-5, Supelco, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The column temperature was set at 130°C for 2 min then increased to 270°C (rate: 30°C/min) for 10 min and was augmented again to 290°C (rate: 10°C/min). After 8 min the temperature was increased to 310°C (rate: 20°C/min) for 2 min, (total time was 30 min). The injector and detector temperatures were set at 295°C and 300°C, respectively. The carrier gas (helium) flow rate was set for 1 ml/min with the inlet splitter set at 100:1. Peaks of interest were identified using authentic standards (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada).

3.3.6 Assessment of cholesterol synthesis

As described above, plasma markers of cholesterol synthesis (desmosterol, lathosterol) were measured by GC. Subsequently, based on the previously established methods, a surrogate method was used to determine cholesterol synthesis (20, 21). Briefly, the ratio of absolute amount of lathosterol to cholesterol (µmol/mmol of cholesterol) was used to determine cholesterol biosynthesis (22-24).

3.3.7 Plasma fat-soluble vitamin and carotenoid analyses

An isocratic high-performance liquid chromatography HPLC (1100 HPLC, Agilent Technologies, Palo Alto, CA, USA) method was used for simultaneous determination of plasma α-tocopherol, γ-tocopherol, retinol, lutein, lycopene, and β-carotene. The extraction procedure was as described by Gueguen et al (25). In brief, an internal standard, a solution of retinol acetate and β-apo-8'-carotenal in methanol, was added to 200 uL serum to quantify vitamin and carotenoid levels, respectively. Serum samples were deproteinized with ethanol and extracted twice with hexane. Duplicates of each sample were prepared, then resulting extracts injected onto a C18 reversed-phase column (Zorbax EclipseXDB, Agilent Technologies, Palo Alto, CA, USA) with column guard eluted with methanol-acetonitrile-tetrahydrofuran (75:20:5, v/v/v). Full elution of all the analytes was realized isocratically within 38 min. The detection wavelengths were set at 290 nm and 450 nm for α and γ-tocopherol and the carotenoids analyses, respectively.

3.3.8 Statistical analyses

Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). For all data, baseline and endpoint values were reported as averages of days 1 and 2, and days 28 and 29, respectively. Results are expressed as mean $\pm$ standard error of the mean (SEM). Baseline, endpoint, absolute change, and percentage change values were compared using a crossover analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test, with treatment and phase as fixed factors, and subject as a random factor in the model. The effect of potential sources of bias was examined including starting lipid values, treatment order, response to specific treatment, and

seasonal effect through the trial. No bias was identified, which indicated that the sample size was representative of the multiple possible responses to be found within the population. Baseline values were inserted into the model as covariates for serum lipid measurements. To standardize plasma PS, as well as serum fat-soluble vitamins and carotenoids, serum concentrations of each compound were divided by plasma TC or LDL-C concentrations, respectively. For serum lipid levels, a two-tailed paired Student's test was used to compare baseline and endpoint within each treatment. Statistical significance was set at $p<0{\cdot}05$ for all analyses.

3.4 Results

3.4.1 Subject characteristics

Fifty-three participants were initially recruited while 25 males and 22 females completed the entire trial. Reasons for the inability to complete the trial included not being able to consume yogurt (n=2), relocation to another city (n=1), and personal reasons (n=3). Baseline characteristics of the subjects who completed the study are shown in Table 2. All participants exhibited good tolerance to the experimental treatments and no side effects were reported. The subjects reported no change in physical activity, and no significant differences were observed in body weight after the consumption of the three treatments.

3.4.2 Serum lipid concentrations in response to PS treatments

Baseline and endpoint plasma lipid concentration responses to each treatment are shown in Table 3. No significant differences at baseline among the three treatments in any of the lipid parameters assessed were observed.

Supplementation of both WD-PS and PS-esters improved serum lipoprotein profiles. In particular, reductions ($p< 0.001$) were observed in endpoint total and LDL cholesterol levels compared with control. Although no treatment effects were observed in HDL-C levels at endpoint across the three treatments, ratios of TC to HDL-C and non-HDL-C to HDL-C showed significant reductions (10.5%, 15.2%, $p<0.05$, respectively) for the supplementation of WD-PS, but not for supplementation of the PS-esters compared to control (Table 3). Plasma TG concentrations failed to as a function of treatment between endpoints. Over the treatment period, however, TG levels were reduced (13.9%, $p<0.05$) from baseline due to consumption of WD-PS-enriched yogurt, compared to PS-esters-enriched yogurt, but not compared to control (Table 3).

Finally, compared with the control product, consumption of the WD-PS-enriched yogurt and PS esters-enriched yogurt similarly lowered ($p<0.001$ for all) TC concentrations on average by 7.7 % and 6.2%, respectively. Likewise, consumption of WD-PS and PS-esters in yogurt lowered ($p<0.001$ for all) LDL-C on average by 11.7% and 11.6%, respectively (Table 3). No differences were observed in baseline to endpoint of body weights across the three treatments. Furthermore, initial BMI was not associated with

subsequent reductions in TC and LDL-C levels in response to PS-esters and/or dispersible PS supplementation.

3.4.3 Plasma carotenoid and vitamin levels in response to treatment

Table 4 provides the fat-soluble vitamin and carotenoid concentrations at the end of each phases, as well as the serum fat-soluble vitamin and carotenoid concentrations, expressed relative to LDL-C levels. Neither intervention, WD-PS and/or PS-esters, had an effect on neither fat-soluble vitamins nor carotenoid levels. Although supplementation of WD-PS and PS-esters slightly decreased serum α-tocopherol and γ-tocopherol concentrations, no significant differences were observed for these two vitamins relative to control. Moreover, after α-tocopherol and γ-tocopherol concentrations were corrected for LDL-C levels, no significant differences were found across treatments (Table 4). Likewise, consumption of both WD-PS and PS esters-enriched yogurts were followed by slight reductions in concentrations of all plasma carotenoids.

3.4.4 Plasma plant sterol concentrations in response to treatments

Plasma PS concentrations and PS concentrations expressed relative to TC are provided in Table 5 at the end of each phases. Plasma concentrations of β-sitosterol were increased ($p<0.001$) for both WD-PS and PS-esters relative to control. Similarly, endpoint adjusted β-sitosterol by TC levels were increased ($p<0.001$) after both the WD-PS and PS-ester phases, compared to control. Plasma stigmasterol levels similarly increased ($p<0.001$) after consumption of the WD-PS-enriched yogurt compared to control yogurt. Likewise, the endpoint adjusted stigmasterol, to TC, were increased ($p<0.05$) for WD-PS phase, not

for PS-esters, compared to control. For plasma campesterol there was a trend (p=0.12) towards increased concentration in the WD-PS phase compared to control. Nonetheless, after adjusting for TC, plasma levels of campesterol increased (p<0.05) for WD-PS phase compared to control. No effects of WD-PS or PS-esters were observed on the cholesterol synthesis markers desmosterol and lathosterol.

3.4.5 Clinical chemistry measures as a function of treatment

Clinical chemical parameters analyzed include AST, ALT, ALKP, GGT, LDH, and CRP levels (Table 6). Although variations were found in the parameters, these differences were of no statistical or clinical significance. No treatment effect was observed with any of these parameters.

3.5 Discussion

In the present study, supplementation of WD-PS resulted in a reduction of plasma TC and LDL-C concentrations compared to control; supporting the notion that proper solubilization of new formulation of PS is essential for proper efficacy. In addition, consumption of WD-PS-enriched yogurt, but not PS-esters, effectively lowered TC/HDL-C and Non-HDL/HDL-C ratios, compared to control. Considering the importance of the TC/HDL-C ratio, as a better index in identifying individuals associated with greater risk of subsequent coronary heart disease events than TC or LDL-C levels alone (10), the lower TC/HDL-C ratio observed gives an indication of the potential benefits of food fortified with the new formulation of WD-PS in improving plasma lipid profiles in the hyperlipidemic individuals studied.

In accordance with the plasma plant sterols and cholesterol precursor concentrations data, the percent cholesterol synthesis rates did not increase with supplementation of WD-PS or PS-esters, as compared to the placebo phase. Additionally, supplementation of WD-PS or PS-esters failed to adversely influence fat soluble vitamin or carotenoid levels before or after adjustment for LDL-C levels, as compared to the placebo. Furthermore, this study reaffirms that yogurt can be utilized as an efficacious PS delivery vehicle to favorably shift cholesterol concentrations.

Long term consumption of PS has been shown to decrease plasma fat soluble and carotenoid concentrations (26, 27). In the present study no reduction in the concentrations of plasma fat soluble vitamins or carotenoids was observed after consumption of WD-PS or PS-esters compared to control. The present results are in agreement with those of Raeini-Sarjaz et al (28) who showed no effect of sterol (1.9 g/d) or stanol esters (1.8 g/d) enriched diets on serum retinol, α - and γ-tocopherol, vitamin D and K concentrations, or their change relative to baseline. Similarly, Hallikainen and Uusitupa (29) reported no changes in serum retinol concentrations after 8 wk of consumption of 2.3 g/d stanol esters, while serum β-carotene and α-tocopherol concentrations were reduced. Therefore, we can conclude that the 2 g/d dose of WD-PS or PS-esters appear to provide minimal reductions in other plasma fat-soluble components.

In the present study, consumption of PS–enriched yogurt (WD-PS and PS-esters) increased plasma sitosterol levels while no changes were observed in plasma campesterol levels. This finding is inconsistent with existing knowledge that an increase in PS intake

promotes plasma concentrations of sitosterol and campesterol by 20% to 100% and 40% to 100%, respectively (13, 30, 31).

Furthermore, in the present study consumption of WD-PS or PS-esters showed no increase in cholesterol synthesis biomarkers. Similar to this result, Gremaud et al. (10) observed that lecithin-solubilized stanols, in an oil-water emulsion, decreased cholesterol absorption without a corresponding increase in cholesterol synthesis. It should be noted that the increases in cholesterol synthesis, as in this present study, were less extensive compared to effects seen previously (31) which used PS in traditional matrices, such as margarine. The difference in magnitude of the change in cholesterol synthesis may also be due to the dose of PS consumed or to the frequency of PS consumption, either of which could potentially alter cholesterol synthesis to a greater or lesser degree (32).

In summary, our findings demonstrate that consumption of WD-PS enriched yogurt by mild-to-moderately hypercholesterolemic subjects decreased TC/HDL-C and non-HDL/HDL-C ratios, and TG levels to a greater extent than PS-esters consumption. However, both WD-PS and PS-ester- enriched yogurt favorably modify blood lipid profiles without altering plasma liver enzymes and/or CRP concentrations. Hence, our study supports the fact that there may be added advantages of a more highly solubilized WD-PS form over traditional PS-esters in terms of overall lipid level management.

3.6 Acknowledgment

We gratefully acknowledge the participants of the study and also personnel at the Richardson Centre for Functional Foods and Nutraceuticals including Khatima Khalloufi and Haifeng Yang for their assistance as analytical technicians, and Kimberley Robinson for her help as clinical coordinator. The present work was funded by Naturalis S.A. Inc (Santiago, Chile).

The authors' responsibilities were as follows- MAS: was responsible for conducting the research, management of the clinical trial, data collection, laboratory analysis, statistical analysis and writing the manuscript ; SVH: was responsible for subject recruitment, and contributed to conduct the clinical trial, data collection, and to revise the final manuscript; PJHJ: was the principal investigator and responsible for the conception and design of the project, submission for ethical approval, seeking financial support and contribution to the preparation of the manuscript. All the authors reviewed the final version of the manuscript and had no conflicts of interest to declare.

3.7 References

1. WHO. 2011. Cardiovascular Diseases (CVDs), WHO Fact Sheet NO.317, World Health Organization (WHO), Geneva, Switzerland. Available at: <http://www.who.int/mediacentre/factsheets/fs317/en/index.html>.

2. NCEP. 2001. Executive Summary of The Third Report of The National Cholesterol Education Program Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 285:2486-2497.

3. HealthCanada. 2010. Plant Sterols and Blood Cholesterol Lowering. *Health Canada,* Food Directorate, Health Products and Food Branch Ottawa (ON).

4. AmericanDiabetesAssociation. 2008. Nutrition Recommendations and Interventions for Diabetes; A position statement of the American Diabetes Association In Diabetes Care.561-578.

5. Trautwein, E.A., Duchateau, G.S.M.J.E., Lin, Y., Mel'nikov, S.M., Molhuizen, H.O.F., and Ntanios, F.Y. 2003. Proposed mechanisms of cholesterol-lowering action of plant sterols. *European Journal of Lipid Science and Technology* 105:171-185.

6. Abumweis, S.S., Barake, R., and Jones, P.J. 2008. Plant sterols/stanols as cholesterol lowering agents: A meta-analysis of randomized controlled trials. *Food Nutr Res* 52.

7. Berger, A., Jones, P.J., and Abumweis, S.S. 2004. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lipids Health Dis* 3:5.

8. Fornari, T., Torres, C.F., Torrelo, G., Senorans, F.J., and Reglero, G. 2009. Production of phytosterol esters from soybean oil deodorizer distillates. *European Journal of Lipid Science and Technology* 111:459-463.

9. Ostlund, R.E., Jr., Spilburg, C.A., and Stenson, W.F. 1999. Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. *Am J Clin Nutr* 70:826-831.

10. Ballantyne, C.M., and Hoogeveen, R.C. 2003. Role of lipid and lipoprotein profiles in risk assessment and therapy. *Am Heart J* 146:227-233.

11. Lin, X., Ma, L., Racette, S.B., Anderson Spearie, C.L., and Ostlund, R.E., Jr. 2009. Phytosterol glycosides reduce cholesterol absorption in humans. *Am J Physiol Gastrointest Liver Physiol* 296:G931-935.

12. Demonty, I., Ras, R.T., van der Knaap, H.C., Duchateau, G.S., Meijer, L., Zock, P.L., Geleijnse, J.M., and Trautwein, E.A. 2009. Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake. *J Nutr* 139:271-284.

13. Jones, P.J., Raeini-Sarjaz, M., Ntanios, F.Y., Vanstone, C.A., Feng, J.Y., and Parsons, W.E. 2000. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. *J Lipid Res* 41:697-705.

14. Weststrate, J.A., and Meijer, G.W. 1998. Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur J Clin Nutr* 52:334-343.

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

16. Moreau, R.A., Whitaker, B.D., and Hicks, K.B. 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Prog Lipid Res* 41:457-500.

17. Patentscope, W. 2010. (WO2010095067) Dispersion of phytosterols *Available at: http://www.wipo.int/patentscope/search/en/WO2010095067*

18. Friedewald, W.T., Levy, R.I., and Fredrickson, D.S. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18:499-502.

19. Sharrett, A.R., Ballantyne, C.M., Coady, S.A., Heiss, G., Sorlie, P.D., Catellier, D., and Patsch, W. 2001. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. *Circulation* 104:1108-1113.

20. Gordon, D.J., and Rifkind, B.M. 1989. High-density lipoprotein--the clinical implications of recent studies. *N Engl J Med* 321:1311-1316.

21. Mackay, D., and Jones, P.J. 2011. Evaluation of methods for the determination of cholesterol absorption and synthesis in humans. *Atherosclerosis* 218:253-262.

22. Simonen, P., Gylling, H., and Miettinen, T.A. 2008. The validity of serum squalene and non-cholesterol sterols as surrogate markers of cholesterol synthesis and absorption in type 2 diabetes. *Atherosclerosis* 197:883-888.

23. Miettinen, T.A., Strandberg, T.E., and Gylling, H. 2000. Noncholesterol sterols and cholesterol lowering by long-term simvastatin treatment in coronary patients: relation to basal serum cholestanol. *Arterioscler Thromb Vasc Biol* 20:1340-1346.

24. Vanstone, C.A., and Jones, P.J. 2004. Limitations of plasma plant sterols as indicators of cholesterol absorption. *Am J Clin Nutr* 79:340-341.

25. Gordon, D.J., Probstfield, J.L., Garrison, R.J., Neaton, J.D., Castelli, W.P., Knoke, J.D., Jacobs, D.R., Jr., Bangdiwala, S., and Tyroler, H.A. 1989. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* 79:8-15.

26. Hansel, B., Nicolle, C., Lalanne, F., Tondu, F., Lassel, T., Donazzolo, Y., Ferrieres, J., Krempf, M., Schlienger, J.L., Verges, B., et al. 2007. Effect of low-fat, fermented milk enriched with plant sterols on serum lipid profile and oxidative stress in moderate hypercholesterolemia. *Am J Clin Nutr* 86:790-796.

27. Law, M. 2000. Plant sterol and stanol margarines and health. *BMJ* 320:861-864.

28. Raeini-Sarjaz, M., Ntanios, F.Y., Vanstone, C.A., and Jones, P.J. 2002. No changes in serum fat-soluble vitamin and carotenoid concentrations with the intake of plant sterol/stanol esters in the context of a controlled diet. *Metabolism* 51:652-656.

29. Hallikainen MA, U.M. 1999. Effect of 2 low-fat stanol estercontaining margarines on serum cholesterol concentrations as part of low-fat diet in hypercholesterolemic subjects. *Am J Clin Nutr* 69 (3):403-410.

30. Derdemezis, C.S., Filippatos, T.D., Mikhailidis, D.P., and Elisaf, M.S. 2010. Review article: effects of plant sterols and stanols beyond low-density lipoprotein cholesterol lowering. *J Cardiovasc Pharmacol Ther* 15:120-134.

31. Fransen, H.P., de Jong, N., Wolfs, M., Verhagen, H., Verschuren, W.M., Lutjohann, D., von Bergmann, K., Plat, J., and Mensink, R.P. 2007. Customary use of plant sterol and plant stanol enriched margarine is associated with changes in serum plant sterol and stanol concentrations in humans. *J Nutr* 137:1301-1306.

32. Rudkowska, I., AbuMweis, S.S., Nicolle, C., and Jones, P.J. 2008. Association between non-responsiveness to plant sterol intervention and polymorphisms in cholesterol metabolism genes: a case-control study. *Appl Physiol Nutr Metab* 33:728-734.

3.8 Tables

Table 1: Composition of plant sterols

	WD-PS	PS-esters
Beta-sitosterol	70%-80%	<80%
Campesterol	<15%	<30%
Stigmasterol	<2%	<30%
Sitostanol	<15%	<15%
Campestanol	<5%	<5%
Other sterols	<3%	<3%

Note: WD-PS, water dispersible plant sterol; PS-esters, esterified plant sterol

Table 2: Baseline characteristic of subjects

Anthropometric and serum lipid measurements	Mean	SEM
Age (years)	50	2.1
Body wt (kg)	80.4	2.7
Height (cm)	168.2	1.4
BMI (kg/m²)	28.2	0.7
Total cholesterol (mmol/l)	6.0	0.2
LDL-cholesterol (mmol/l)	3.8	0.2
HDL-cholesterol (mmol/l)	1.4	0.1
TG (mmol/l)	1.7	0.1
CRP (mg/l)	1.5	0.3
Blood Pressure:		
Systolic (mmHg)	121	2.4
Diastolic (mmHg)	78	1.8
Liver Enzymes:		
AST (U/l)	22.9	2.0
ALT (U/l)	38.2	4.7
LDH (U/l)	529.7	10.7
ALKP (U/l)	76.9	3.0
GGT (U/l)	37.4	3.4

Note: ALKP, alkaline phosphatase; ALT, alanine aminotransferase;

AST, asparagine aminotransferase; GGT, g-glutamyltransferase;

LDH, lactate dehydrogenase; CRP, C-reactive protein

Table 3: Plasma lipid concentrations in response to each treatments

Parameters	Control	WD-PS yogurt	PS-esters yogurt
Total Cholesterol			
Start	5.85±0.15	5.98±0.15	5.89±0.14
End	6.04±0.15	5.73±0.13**	5.72±0.13**
Change	0.20±0.08	-0.26±0.07**	-0.18±0.08**
Change (%)	3.85 ± 1.25	-3.85 ± 1.12**	-2.41 ± 1.35*
Change relative to control (%)		-7.69 ± 1.46	-6.25 ± 1.93
LDL-Cholesterol			
Start	3.67±0.14	3.84±0.14	3.75±0.13
End	3.82±0.15	3.58±0.13**	3.50±0.12**
Change	0.17±0.08	-0.27±0.06**	-0.25±0.07**
Change (%)	5.5 ± 2.1	-6.22 ± 1.46**	-6.05 ± 1.91**
Change relative to control (%)		-11.72 ± 2.52	-11.56 ± 2.94
Non-HDL Cholesterol			
Start	4.40±0.15	4.58±0.15	4.48±0.14
End	4.60±0.15	4.30±0.13	4.32±0.13
Change	0.20±0.09	-0.28±0.07	-0.17±0.07
Change (%)	5.69 ± 1.96	-5.44 ± 1.42**	-3.03 ± 1.56*
Change relative to control (%)		-11.13 ± 2.34	-8.72 ± 2.60
HDL-Cholesterol			
Start	1.44±0.06	1.40±0.05	1.41±0.05
End	1.44±0.05	1.43±0.05	1.40±0.05
Change	-0.01±0.04	0.03±0.02	-0.02±0.02
Change (%)	1.18 ± 1.85	2.65 ± 1.79	-0.05 ± 1.58
Change relative to control (%)		2.66 ± 1.79	-0.04 ± 1.58
Triglycerides			
Start	1.70±0.12	1.68±0.11	1.63±0.11
End	1.80±0.13	1.62±0.12	1.80±0.13
Change	0.09±0.09	-0.05±0.05 †	0.17±0.06
Change (%)	13.16 ± 7.71	-0.76 ± 3.40†	13.74 ± 4.49
Change relative to control (%)		-13.92 ± 8.63 †	0.58 ± 9.05
TC/HDL-C			
Start	4.32±0.19	4.50±0.18	4.41±0.17
End	4.46±0.19	4.21±0.16*	4.33±0.18
Change	0.13±0.11	-0.30±0.09**	-0.09±0.06
Change (%)	5.16 ± 3.61	-5.39 ± 1.53*	-1.85 ± 1.34
Change relative to control (%)		-10.55 ± 3.75	-7.01 ± 3.97
NHDL/HDL-C			
Start	3.32±0.19	3.5±0.18	3.41±0.17
End	3.46±0.19	3.21±0.16	3.32±0.18
Change	0.13±0.11	-0.29±0.09**	0.09±0.06
Change (%)	8.47± 5.83	-6.74 ± 1.94*	-2.36 ± 1.74
Change relative to control (%)		-15.21 ± 5.90	-10.82 ± 6.23

Note: Concentrations are expressed as mmol/l, except for the ratios.

Treatment effects were examined by one-way ANOVA. Values are mean±SEM; N=47.

(*p<0.05, **p<0.001) Comparisons of WD-PS and PS-esters with Control are significant.

† p<0.05 Comparison of WD-PS with PS-esters is significant, but not significant from Control.

Change and percentage change are based on individual data.

Table 4: Endpoint plasma carotenoid and vitamin levels in response to treatment

	Control	WD-PS		PS-esters	
	Wk 4	Wk 4	Absolute change	Wk 4	Absolute change
α-Tocopherol	63.87±2.64	62.66±2.51	-1.22±3.28	62.36±4.11	-1.51±3.28
α-Tocopherol-LDL	10.54±0.43	10.9±0.45	0.37±0.51	10.79±0.63	0.25±0.51
γ-Tocopherol	9.62±0.65	9.87±0.72	0.25±0.84	9.61±0.41	-0.30±0.84
γ-Tocopherol/LDL	1.60±0.11	1.73±0.13	0.13±0.13	1.62±0.07	0.03±0.13
Retinol	3.40±0.19	3.88±0.18	-0.02±0.19	3.89±0.26	-0.00±0.19
Retinol/LDL	0.65±0.03	0.67±0.03	0.02±0.03	0.67±0.04	0.03±0.03
Lutein	0.36±0.02	0.36±0.02	0.00±0.02	0.32±0.02	-0.04±0.02
Lutein/LDL	0.06±0.00	0.06±000	0.00±0.00	0.06±0.00	-0.00±0.00
β.Carotene	0.63±0.07	0.62±0.05	-0.01±0.05	0.59±0.06	-0.04±0.05
β.Carotene/LDL	0.10±0.00	0.10±0.00	0.00±0.01	0.10±0.00	0.00±0.01
Lycopene	0.69±0.05	0.62±0.04	-06±0.07	0.63±0.07	-0.06±0.07
Lycopene/LDL	0.12±0.00	0.11±0.00	-0.01±0.01	0.11±0.01	0.01±0.01

Note: Concentrations are expressed as µmol/l Values are mean± SEM; N=47.

Corrected values to LDL are expressed as µmol/mmol.

Treatment effects were examined at the end of the three different phases by one-way ANOVA.

Absolute change was calculated for each subject as follow: [Wk 4 (WD-PS, PS-esters)]-[Wk 4 (control)].

Table 5: Plasma plant sterol, stanol, and cholesterol precursor concentrations at the end of each treatment phases

	Control	WD-PS		PS-esters	
	Wk 4	Wk 4	Absolute change	Wk 4	Absolute change
Desmosterol	2.80±0.37	2.95±0.35	0.15±0.33	3.06±0.29	0.26±0.33
Brassicasterol	0.18±0.14	0.22±0.09	0.03±0.15	0.19±0.09	0.01±0.15
Lathosterol	3.69±0.36	4.03±0.37	0.34±0.27	3.86±0.36	0.18±0.27
Campesterol	6.47±0.62	7.04±0.7	0.58±0.37	6.75±0.37	0.25±0.37
Stigmasterol	0.11±0.06	0.65±0.28	0.54±0.26**	0.30±0.15	0.18±0.26
β sitosterol	3.52±0.47	6.06±0.62	2.55±0.46**	5.51±0.56	2.00±0.46**
Stigmastanol	0.35±0.14	0.58±0.46	0.23±0.41	0.49±0.33	0.14±0.41
Ratios (µmol/mmol)					
Desmosterol/Cholesterol	0.46±0.06	0.51±0.06	0.05±0.06	0.54±0.05	0.07±0.06
Brassicasterol/Cholesterol	0.02±0.02	0.04±0.02	0.13±0.02	0.03±0.02	0.01±0.02
Lathosterol/Cholesterol	0.61±0.06	0.70±0.06	0.09±0.05	0.67±0.06	0.06±0.05
Campesterol/Cholesterol	1.07±0.10	1.23±0.12	0.15±0.06*	1.17±0.11	0.10±0.06
Stigmasterol/Cholesterol	0.02±0.01	0.12±0.05	0.10±0.05*	0.06±0.03	0.04±0.05
β sitosterol/Cholesterol	0.58±0.08	1.05±0.10	0.47±0.14**	0.96±0.10	0.37±0.13**
Stigmastanol/Cholesterol	0.06±0.02	0.10±0.08	0.04±0.07	0.08±0.06	0.03±0.07

Note: Concentrations are expressed as µmol/l Values are mean±SEM; N=47

Treatment effects were examined at the end of the three different phases by one-way ANOVA.

Absolute change was calculated for each subject as follow: Wk 4 (WD-PS, PS-esters)]-[Wk 4 (control)].

**p<0.001 compared with the control *p<0.05 compared with control.

Table 6: Liver function test parameters and C- reactive protein levels in response to treatments

	Control	WD-PS		PS-esters	
	Wk 4	Wk 4	Absolute change	Wk 4	Absolute change
AST (U/l)	20.6±1.33	22.5±1.72	1.9±1.30	21.3±1.48	0.7±1.30
ALT (U/l)	35.2±2.71	38.1±3.54	2.9±3.13	40.5±4.54	5.4±3.13
LDH (U/l)	504.5±7.9	509.7±9.9	5.2±11.02	514.8±13.17	10.4±11.02
ALKP (U/l)	80.3±6.39	73.7±2.52	-6.6±6.93	72.8±2.62	-7.5±6.93
GGT (U/l)	35.3±2.84	36.8±3.08	1.6±1.51	36.6±2.82	1.5±1.51
CRP (mg/l)	1.75±0.35	1.50±0.29	-0.26±0.44	1.60±0.31	-0.15±0.44

Note: Values are means±SEM.

ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, asparagine aminotransferase

GGT, gamma-glutamyltransferase; LDH, lactate dehydrogenase; CRP, C-reactive protein

Absolute change was calculated for each subject as follow: Wk 4 (WD-PS, PS-esters)]-[Wk 4 (control)].

CHAPTER 4. SUMMARY OF FINDINGS AND GENERAL CONCLUSION

The purpose of this study was to assess the LDL-C lowering efficacy of a new formulation of water dispersible plant sterol, compared to plant sterol esters, in treatment of dyslipidemia. The following section outlines key points of the findings, discusses their implication, and gives suggestions for future research in this area.

In reviewing the data on two different formulations of PS (WD-PS vs. PS-esters), it was shown that supplementation of WD-PS not only helps to regulate circulating total and LDL-C levels equally to PS-esters, but is also associated with additional benefits for optimizing lipid profiles. More specifically, supplementation of WD-PS lowered ratios of TC to HDL-C and also NHDL-C to HDL-C while consumption of PS-esters did not effectively alter any of these two ratios. In addition, it was shown that consumption of WD-PS resulted in a decrease of TG levels compared to PS-ester.

Accurate risk assessment requires a complete lipid profile because it will identify more high-risk individuals than evaluating LDL-C levels alone (50). Although lowering LDL-C remains to be the first line therapy for primary and secondary prevention CHD, the latest guidelines of the third Adult Treatment Panel guidelines of the US National Cholesterol Education Program (ATP III) recommend a full fasting lipoprotein profile to include total cholesterol, LDL-C, HDL-C, and triglyceride levels, as the initial lipid measurement in all individuals for CHD risk assessment (64).

As in risk assessment, there is much evidence to suggest that elevated triglycerides are predictive of CHD risk (65, 66), however, it is often accompanied by other metabolic disturbances including reduced levels of HDL-C, and elevated TC and TC/HDL-C ratio which amplify CHD risk. While the interaction with these predictive parameters of CHD risk makes it difficult to assess the independent risk conferred by TG levels, combined elevations of TG and LDL-C levels may confer greater risk than isolated increases in either one (50, 67).

More recently, the ratio of TC/HDL-C as an index combining the proportion of atherogenic to antiatherogenic lipids and lipoproteins, has been considered not only as the best predictor of CHD risk in epidemiologic studies (50), but also as having value as a therapeutic target and recognized as a predictor for cardiovascular risk in patients on LDL-C–lowering therapy (47, 67). Global cardiovascular risk assessment described in the 2009 recommendations from the Canadian Cardiovascular Society, set target lipid levels to better stratify intermediate risk patients (68). In the guidelines, reaching LDL-C goals remains to be the first line therapy. TC/HDL-C ratio and triglyceride levels are also defined as important in risk stratification in primary prevention of CHD with the defined optional target as TC/HDL-C <4 and TG <1.7 mmol/L).

Thus, based on the demonstrated importance of T-C/HDL-C ratio (46, 69, 70) as a better index to predict the risk of heart disease than traditional lipid testing indicators, TC alone or LDL-C levels; the lower T-C/HDL-C and NHDL-C/HDL-C ratios observed after consumption of WD-PS may confirm that this new formulation is a more highly

advantageous form of PS for hypercholesterolemia than PS-esters. Moreover, the lower TG levels observed after consumption of WD-PS, compared to PS-esters, gives an indication of the potential improved benefits of food fortified with WD-PS in prevention of CHD in moderate to hyperlipidemic individuals.

Moreover, in most jurisdictions, public health recommendations for healthy eating suggest reduction in the total amount of fat consumed by all individuals. Accordingly, current community preference lies in the direction of reduced fat intake that may reduce consumption of high fat fortified food with plant sterol including margarines, while WD-PS is not require to be incorporated in fat based foods. Thus, it is expected that long-term compliance of fat free foods fortified with WD-PS remains significant.

In conclusion, these findings demonstrated that the consumption of yogurt fortified with the new formulation of WD-PS in moderate to hypercholesterolemic subjects, in the context of free-living, favorably modulates certain indicators of CHD risk. In particular, TC and LDL-C levels were improved by a comparable degree for WD-PS and PS-esters. However, TG levels, as well as TC/HDL-C and non-HDL-C/HDL-C ratio concentrations were decreased as a result of supplementation of WD-PS, but not for PS-esters. In view of these findings, it is possible to support the use of a new formulation of WD-PS as an effective nutraceutical means of improving lipid profiles, which may offer a greater level of heart disease protection compared to PS-esters in moderate to hypercholesterolemic population.

BIBLIOGRAPHY:

1. Ostlund, R.E., Jr. 2002. Phytosterols in human nutrition. *Annu Rev Nutr* 22:533-549.

2. NCEP. 2001. Executive Summary of The Third Report of The National Cholesterol Education Program Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 285:2486-2497.

3. Health Canada. 2010. Plant Sterols and Blood Cholesterol Lowering, Health Canada, Food Directorate, Health Products and Food Branch, Bureau of Nutritional Sciences,. Ottawa (ON).

4. American Diabetes Association. 2008. Nutrition Recommendations and Interventions for Diabetes A position statement of the American Diabetes Association In Diabetes Care. 561-578.

5. Katan, M.B., Grundy, S.M., Jones, P., Law, M., Miettinen, T., and Paoletti, R. 2003. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin Proc* 78:965-978.

6. Garcia-Llatas, G., and Rodriguez-Estrada, M.T. 2011. Current and new insights on phytosterol oxides in plant sterol-enriched food. *Chem Phys Lipids* 164:607-624.

7. Calpe-Berdiel, L., Escola-Gil, J.C., and Blanco-Vaca, F. 2009. New insights into the molecular actions of plant sterols and stanols in cholesterol metabolism. *Atherosclerosis* 203:18-31.

8. Weingartner, O., Bohm, M., and Laufs, U. 2009. Controversial role of plant sterol esters in the management of hypercholesterolaemia. *Eur Heart J* 30:404-409.

9. Moreau, R.A., Whitaker, B.D., and Hicks, K.B. 2002. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Prog Lipid Res* 41:457-500.

10. Othman, R.A., and Moghadasian, M.H. 2011. Beyond cholesterol-lowering effects of plant sterols: clinical and experimental evidence of anti-inflammatory properties. *Nutr Rev* 69:371-382.

11. Noakes, M., Clifton, P.M., Doornbos, A.M., and Trautwein, E.A. 2005. Plant sterol ester-enriched milk and yoghurt effectively reduce serum cholesterol in modestly hypercholesterolemic subjects. *Eur J Nutr* 44:214-222.

12. Hendriks, H.F., Brink, E.J., Meijer, G.W., Princen, H.M., and Ntanios, F.Y. 2003. Safety of long-term consumption of plant sterol esters-enriched spread. *Eur J Clin Nutr* 57:681-692.

13. Hendriks, H.F., Weststrate, J.A., van Vliet, T., and Meijer, G.W. 1999. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur J Clin Nutr* 53:319-327.

14. Hallikainen, M.A., Sarkkinen, E.S., and Uusitupa, M.I. 2000. Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner. *J Nutr* 130:767-776.

15. Maki, K.C., Davidson, M.H., Umporowicz, D.M., Schaefer, E.J., Dicklin, M.R., Ingram, K.A., Chen, S., McNamara, J.R., Gebhart, B.W., Ribaya-Mercado, J.D., et al. 2001. Lipid responses to plant-sterol-enriched reduced-fat spreads incorporated into a National Cholesterol Education Program Step I diet. *Am J Clin Nutr* 74:33-43.

16. Law, M. 2000. Plant sterol and stanol margarines and health. *BMJ* 320:861-864.

17. AbuMweis, S.S., Barake, R., and Jones, P.J.H. 2008. Plant sterols/stanols as cholesterol lowering agents: a meta-analysis of randomized controlled trials *Food and Nutrition Research* DOI: 10.3402/fnr.v52i0.1811.

18. Demonty, I., Ras, R.T., van der Knaap, H.C., Duchateau, G.S., Meijer, L., Zock, P.L., Geleijnse, J.M., and Trautwein, E.A. 2009. Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake. *J Nutr* 139:271-284.

19. Altmann, S.W., Davis, H.R., Jr., Zhu, L.J., Yao, X., Hoos, L.M., Tetzloff, G., Iyer, S.P., Maguire, M., Golovko, A., Zeng, M., et al. 2004. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* 303:1201-1204.

20. Turley, S.D. 2008. The role of Niemann-Pick C1 - Like 1 (NPC1L1) in intestinal sterol absorption. *J Clin Lipidol* 2:S20-S28.

21. Rozner, S., and Garti, N. . 2006. The activity and absorption relationship of cholesterol and phytosterols *Colloid Surf A-Physicochem Eng Asp* 282:435-456.

22. AbuMweis, S.S., Vanstone, C.A., Lichtenstein, A.H., and Jones, P.J. 2009. Plant sterol consumption frequency affects plasma lipid levels and cholesterol kinetics in humans. *Eur J Clin Nutr* 63:747-755.

23. Ikeda, I., and Sugano, M. 1983. Some aspects of mechanism of inhibition of cholesterol absorption by beta-sitosterol. *Biochim Biophys Acta* 732:651-658.

24. Ikeda, I., Tanabe, Y., and Sugano, M. 1989. Effects of sitosterol and sitostanol on micellar solubility of cholesterol. *J Nutr Sci Vitaminol (Tokyo)* 35:361-369.

25. Mel'nikov, S.M., Seijen ten Hoorn, J.W., Eijkelenboom, A.P. . 2004. Effect of phytosterols and phytostanols on the solubilization of cholesterol by dietary mixed micelles: an in vitro study. . *Chem Phys Lipids* 127:121-141.

26. Ikeda, I., Tanaka, K., Sugano, M., Vahouny, G.V., and Gallo, L.L. 1988. Inhibition of cholesterol absorption in rats by plant sterols. *J Lipid Res* 29:1573-1582.

27. Armstrong, M.J., and Carey, M.C. 1987. Thermodynamic and molecular determinants of sterol solubilities in bile salt micelles. *J Lipid Res* 28:1144-1155.

28. Ikeda, I., Tanaka, K., Sugano, M., Vahouny, G.V., and Gallo, L.L. 1988. Discrimination between cholesterol and sitosterol for absorption in rats. *J Lipid Res* 29:1583-1591.

29. AbuMweis, S.S., and Jones, P.J. 2008. Cholesterol-lowering effect of plant sterols. *Curr Atheroscler Rep* 10:467-472.

30. Davis, H.R., Jr., Zhu, L.J., Hoos, L.M., Tetzloff, G., Maguire, M., Liu, J., Yao, X., Iyer, S.P., Lam, M.H., Lund, E.G., et al. 2004. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J Biol Chem* 279:33586-33592.

31. Davis Jr, H.R.A., Scott W. 2009. Niemann-Pick C1 Like 1 (NPC1L1) an intestinal sterol transporter. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1791:679-683.

32. Berge, K.E., Tian, H., Graf, G.A., Yu, L., Grishin, N.V., Schultz, J., Kwiterovich, P., Shan, B., Barnes, R., and Hobbs, H.H. 2000. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 290:1771-1775.

33. Heinemann, T., Axtmann, G., and von Bergmann, K. 1993. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur J Clin Invest* 23:827-831.

34. Salen, G., Shore, V., Tint, G.S., Forte, T., Shefer, S., Horak, I., Horak, E., Dayal, B., Nguyen, L., Batta, A.K., et al. 1989. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. *J Lipid Res* 30:1319-1330.

35. Yu, L., Hammer, R.E., Li-Hawkins, J., Von Bergmann, K., Lutjohann, D., Cohen, J.C., and Hobbs, H.H. 2002. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci U S A* 99:16237-16242.

36. Duan, L.P., Wang, H.H., and Wang, D.Q. 2004. Cholesterol absorption is mainly regulated by the jejunal and ileal ATP-binding cassette sterol efflux transporters Abcg5 and Abcg8 in mice. *J Lipid Res* 45:1312-1323.

37. Field, F.J., and Mathur, S.N. 1983. Regulation of acyl CoA:cholesterol acyltransferase by 25-hydroxycholesterol in rabbit intestinal microsomes and absorptive cells. *J Lipid Res* 24:1049-1059.

38. Field, F.J., and Mathur, S.N. 1983. beta-sitosterol: esterification by intestinal acylcoenzyme A: cholesterol acyltransferase (ACAT) and its effect on cholesterol esterification. *J Lipid Res* 24:409-417.

39. Jones, P.J., and AbuMweis, S.S. 2009. Phytosterols as functional food ingredients: linkages to cardiovascular disease and cancer. *Curr Opin Clin Nutr Metab Care* 12:147-151.

40. Ostlund, R.E., Jr. 2007. Phytosterols, cholesterol absorption and healthy diets. *Lipids* 42:41-45.

41. Abumweis, S.S., Barake, R., and Jones, P.J. 2008. Plant sterols/stanols as cholesterol lowering agents: A meta-analysis of randomized controlled trials. *Food Nutr Res* 52.

42. Berger, A., Jones, P.J., and Abumweis, S.S. 2004. Plant sterols: factors affecting their efficacy and safety as functional food ingredients. *Lipids Health Dis* 3:5.

43. Jones, P.J., Ntanios, F.Y., Raeini-Sarjaz, M., and Vanstone, C.A. 1999. Cholesterol-lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men. *Am J Clin Nutr* 69:1144-1150.

44. Denke, M.A. 1995. Lack of efficacy of low-dose sitostanol therapy as an adjunct to a cholesterol-lowering diet in men with moderate hypercholesterolemia. *Am J Clin Nutr* 61:392-396.

5. Trautwein, E.A., Duchateau, G.S.M.J.E., Lin, Y., Mel'nikov, S.M., Molhuizen, H.O.F., and Ntanios, F.Y. 2003. Proposed mechanisms of cholesterol-lowering action of plant sterols. *European Journal of Lipid Science and Technology* 105:171-185.

46. Kannel, W.B., and Wilson, P.W. 1992. Efficacy of lipid profiles in prediction of coronary disease. *Am Heart J* 124:768-774.

47. Kastelein, J.J., van der Steeg, W.A., Holme, I., Gaffney, M., Cater, N.B., Barter, P., Deedwania, P., Olsson, A.G., Boekholdt, S.M., Demicco, D.A., et al. 2008. Lipids, apolipoproteins, and their ratios in relation to cardiovascular events with statin treatment. *Circulation* 117:3002-3009.

48. D'Agostino, R.B., Kannel, W.B., Stepanians, M.N., and D'Agostino, L.C. 1992. Efficacy and tolerability of lovastatin in elderly hypercholesterolemic patients. *Clin Ther* 14:68-76.

49. Ostlund, R.E., Jr., Spilburg, C.A., and Stenson, W.F. 1999. Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. *Am J Clin Nutr* 70:826-831.

50. Ballantyne, C.M., and Hoogeveen, R.C. 2003. Role of lipid and lipoprotein profiles in risk assessment and therapy. *Am Heart J* 146:227-233.

51. Lin, X., Ma, L., Racette, S.B., Anderson Spearie, C.L., and Ostlund, R.E., Jr. 2009. Phytosterol glycosides reduce cholesterol absorption in humans. *Am J Physiol Gastrointest Liver Physiol* 296:G931-935.

52. Kobayashi, M., Hamada, T., Goto, H., Imaizumi, K., and Ikeda, I. 2008. Comparison of effects of dietary unesterified and esterified plant sterols on cholesterol absorption in rats. *J Nutr Sci Vitaminol (Tokyo)* 54:210-214.

53. Meijer, G.W., Bressers, M.A., de Groot, W.A., and Rudrum, M. 2003. Effect of structure and form on the ability of plant sterols to inhibit cholesterol absorption in hamsters. *Lipids* 38:713-721.

54. Nissinen, M.J., Vuoristo, M., Gylling, H., and Miettinen, T.A. 2007. Respective hydrolysis and esterification of esterified and free plant stanols occur rapidly in human intestine after their duodenal infusion in triacyl- or diacylglycerol. *Lipids* 42:603-612.

55. Richelle, M., Enslen, M., Hager, C., Groux, M., Tavazzi,I., Godin, J. P., Berger, A., Métairon, S., Quaile, S., Piguet-Welsch, C., Sagalowicz, L., Green, H., and Fay, L. B. 2004. Both free and esterified plant sterols reduce cholesterol

absorption and the bioavailability of β-carotene and α-tocopherol in normocholesterolemic humans1,2. *Am J Clin Nutr* 80:171-177.

56. Vanstone, C.A., Raeini-Sarjaz, M., Parsons, W.E., and Jones, P.J. 2002. Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons. *Am J Clin Nutr* 76:1272-1278.

57. Korpela, R., Tuomilehto, J., Hogstrom, P., Seppo, L., Piironen, V., Salo-Vaananen, P., Toivo, J., Lamberg-Allardt, C., Karkkainen, M., Outila, T., et al. 2006. Safety aspects and cholesterol-lowering efficacy of low fat dairy products containing plant sterols. *Eur J Clin Nutr* 60:633-642.

58. Clifton, P.M., Noakes, M., Sullivan, D., Erichsen, N., Ross, D., Annison, G., Fassoulakis, A., Cehun, M., and Nestel, P. 2004. Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal. *Eur J Clin Nutr* 58:503-509.

59. Sehayek, E., and Breslow, J.L. 2005. Plasma plant sterol levels: another coronary heart disease risk factor? *Arterioscler Thromb Vasc Biol* 25:5-6.

60. Chan, Y.M., Varady, K.A., Lin, Y., Trautwein, E., Mensink, R.P., Plat, J., and Jones, P.J. 2006. Plasma concentrations of plant sterols: physiology and relationship with coronary heart disease. *Nutr Rev* 64:385-402.

61. Hansel, B., Nicolle, C., Lalanne, F., Tondu, F., Lassel, T., Donazzolo, Y., Ferrieres, J., Krempf, M., Schlienger, J.L., Verges, B., et al. 2007. Effect of low-fat, fermented milk enriched with plant sterols on serum lipid profile and oxidative stress in moderate hypercholesterolemia. *Am J Clin Nutr* 86:790-796.

62. Fransen, H.P., de Jong, N., Wolfs, M., Verhagen, H., Verschuren, W.M., Lutjohann, D., von Bergmann, K., Plat, J., and Mensink, R.P. 2007. Customary use of plant sterol and plant stanol enriched margarine is associated with changes in serum plant sterol and stanol concentrations in humans. *J Nutr* 137:1301-1306.

63. Derdemezis, C.S., Filippatos, T.D., Mikhailidis, D.P., and Elisaf, M.S. 2010. Review article: effects of plant sterols and stanols beyond low-density lipoprotein cholesterol lowering. *J Cardiovasc Pharmacol Ther* 15:120-134.

64. NCEP. 2001. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 285:2486-2497.

65. Sharrett, A.R., Ballantyne, C.M., Coady, S.A., Heiss, G., Sorlie, P.D., Catellier, D., and Patsch, W. 2001. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and

HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. *Circulation* 104:1108-1113.

66. Castelli, W.P., Anderson, K., Wilson, P.W., and Levy, D. 1992. Lipids and risk of coronary heart disease. The Framingham Study. *Ann Epidemiol* 2:23-28.

67. Ridker, P.M., Rifai, N., Cook, N.R., Bradwin, G., and Buring, J.E. 2005. Non-HDL cholesterol, apolipoproteins A-I and B100, standard lipid measures, lipid ratios, and CRP as risk factors for cardiovascular disease in women. *JAMA* 294:326-333.

68. Genest, J., McPherson, R., Frohlich, J., Anderson, T., Campbell, N., Carpentier, A., Couture, P., Dufour, R., Fodor, G., Francis, G.A., et al. 2009. 2009 Canadian Cardiovascular Society/Canadian guidelines for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease in the adult - 2009 recommendations. *Can J Cardiol* 25:567-579.

69. Gordon, D.J., Probstfield, J.L., Garrison, R.J., Neaton, J.D., Castelli, W.P., Knoke, J.D., Jacobs, D.R., Jr., Bangdiwala, S., and Tyroler, H.A. 1989. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* 79:8-15.

70. Grover, S.A., Palmer, C.S., and Coupal, L. 1994. Serum lipid screening to identify high-risk individuals for coronary death. The results of the Lipid Research Clinics prevalence cohort. *Arch Intern Med* 154:679-684.

APPENDIX

Appendix1. Human ethics approval form

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

RECEIVED
JUL 23 2010

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Naturalis S.A.

Ethics Reference Number: B2010:096
Date of REB Meeting: June 28, 2010
Date of Approval: July 20, 2010
Date of Expiry: June 28, 2011

Protocol Title: Naturalis Clinical Trial for a Novel Phytosterol Formulation

The following is/are approved for use:

- **Protocol, Version dated April 23, 2010**
- **Research Subject Information and Consent Form, Version dated 07/12/2010**
- **Novel Phytosterol Formulation Study Screening Information Form 2010, Version dated 05/10/2010**
- **3 Day Food Diary submitted June 10, 2010**
- **Advertisement submitted June 10, 2010**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated July 14, 2010. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations of Canada.*

This approval is valid for one year from the date of the meeting at which it was reviewed. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone**: (204) 789-3255/ **Fax:** (204) 789-3414

www.umanitoba.ca/faculties/medicine/research/ethics

Appendix2. Medical examination form

Phase Pre-study	**Study Physician**	
Date of Visit ____/____/__ MM DD YR	**Investigator** Dr. Peter Jones	**Subject Code**

COMPLETE PHYSICAL EXAMINATION

A. Vital Signs

Body Weight:_____ lbs _____ _____ kg **Height**: _____ ____cm

Respirations:_____

Blood Pressure (seated): _____/_____ mmHg **Heart Rate**:_____bpm
systolic diastolic

Race/Ethnic Origin:
□ **Caucasian** □ **African-American/Canadian** □ **Asian** □ **Other:**_____

B. Body Systems (Check the appropriate box if organ system was examined. If not done, write N/D in the box)

	Normal	Abnormal	*Details of abnormal finding
1) Ears, Nose, Throat			
2) Eyes			
3) Dermatological			
4) Musculoskeletal			
5) Lymph Nodes			
6) Neurological			
7) Cardiovascular			
8) Respiratory			
9) Endocrine			
10) Urogenital			
11) Gastrointestinal (complete section C)			

C. Gastrointestinal Cont…

Bowel Habits: Frequency_____/Day **Urination:** Frequency_____/Day

Consistency_____	Nocturia_____/Night
Medications: Hospitalizations: Family History:	

D. Medical History		
	YES	**NO**
Have you taken a glucose lowering medication or a medication affecting lipid metabolism (cholestyramine, colestipol, niacin, colfibrate, gemfibrozil, probucol, HMG-CoA reductase inhibitors, and high-dose dietary supplements, plant sterols or fish oil capsules) within the past 3 months?		
Do you take systemic aspirin, NSAIDS. antibodies, corticosteroids, androgens or phenytoin within the past 3 months?		
Are you on anticoagulant therapy?		
Do you smoke?		
Do you consume large amounts of alcohol? (more than 2 drinks per day or 12 drinks per week) Do you follow a specific diet?		
Do you have major food allergy?		
Do you have lactose intolerance?		
Have you had major surgery in the last 6 months?		
Do you have diabetes mellitus?		
Do you have kidney disease?		
Do you have liver disease?		
Do you have heart disease?		

Do you have gastrointestinal, pancreatitis or biliary disease (onset within past three months)?		
Have you had cancer? If yes, occurrence of therapy within past 1 year?		
Do you have anemia, bleeding disorder or significant blood loss/donation?		
Do you have uncontrolled thyroid disease or hypertension? (Subject will be accepted if she is on a stable dose of a thyroid or blood pressure medication that has no known effects on blood lipid metabolism.)		
Do you have a history of eating disorders?		

E. Additional Physician Notes
Based on the medical examination and medical history, is the subject eligible to participate in the study protocol (circle one): **YES** **NO**
Physician's Signature:_____ Date:_____

Appendix 3. General information sheet

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-8883
Fax (204) 474-7552
peter_jones@umanitoba.ca

July 26, 2010 **Unofficial General Information Sheet**

Efficacy of a Plant Sterol-Fortified Dairy Product on Plasma Lipid and Plant Sterol Concentrations in Humans

Study Objective:

The purpose of the study is to evaluate the relative lipid-lowering efficacy of consumption of two different plant sterol (PS) preparations (dispersible free PS compare to PS esters). In particular the study will assess if improved matrixing and dispersion of PS in an emulsion will increase it efficacy in cholesterol lowering. It has been shown that the consumption of PS favourably alter blood cholesterol level. It is anticipated that consumption of the free dispersible PS enriched dairy products (new formulation) will more improve lipid profile, as well as other health-related markers.

Study Design:

The study will consist of 3 treatment phases (30 days each) separately by one month break period. . The 3 phases of treatments will include:

1) Study meal with placebo (regular dairy product).
2) Study meal with free PS dairy product (1.95g/d of PS).
3) Study meal with PS ester-fortified dairy product (1.95g/d of PS).

While participating in the treatment phase, you will be required to come to the RCFFN centre to consume your supper time meal (1 meal) along with your assigned treatment under the supervision of the research staff. The meal will reflect a standard North American diet including foods such as chicken, spaghetti, and chili. You will follow your normal eating routine for the other 2 meals of the day.
At the end of each phase, during the month of break period, you will return to your normal eating and will not have to come to the centre.
We ask you not to consume alcohol or caffeinated beverages throughout the phase**.**

Study Measurements:

1) During days 1, 2, 29 and 30 of each of the three phases of the trial, fasting blood samples (approximately 2 tablespoons will be taken on each blood draw day) will be obtained for assessment of blood fat levels and blood fat metabolism. Each blood test will take

approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 18 tablespoons.
2) On day 1 and 30 of each phase you will be asked to give a urine sample.
3) At the end of each phase you will be asked to fill out a 3-day food diary.
Thank you for considering volunteering for our study.
Please contact Dr. Peter Jones for further information (**204-298-5483)**.

Appendix3. General subject screening form

Richardson Centre for Functional Foods and Nutraceuticals

General Subject Screening Form

To be filled out by participant:
Circle appropriate YES/NO responses

Name:			
Date of Birth:	Month	Day	Year
Sex:	Male:	Female: Postmenopausal: YES NO	
Caucasian	YES NO for Spice trial only		

Contact Information	
Street Address	
Postal Code	
City	
Home Phone:	
Cell Phone:	
Email:	

Medical History			
Diabetes mellitus	YES	NO	If Yes to Other, please specify:
Thyroid disease	YES	NO	
Kidney disease	YES	NO	
Liver disease	YES	NO	
Heart disease	YES	NO	
Hypertension	YES	NO	
Other	YES	NO	

Cholesterol lowering medication? *(in the last 3 months)*		YES	NO
Other medications	YES	NO	If Yes, specify: Are the doses of these medications stable? YES…NO
Vitamin, Mineral supplement	YES	NO	If Yes, specify:
Herbal, food supplement	YES	NO	If Yes, specify:
Laxatives	YES	NO	
Fiber	YES	NO	
Allergies (food such as corn)	YES	NO	If Yes, specify:
Vegetarian	YES	NO	
Any metallic bone components	YES	NO	

Lifestyle		
Smoker?	YES	NO
If Yes, how many per day?		
Drink Alcohol?	YES	NO
If Yes, how many drinks/week		

To be filled out by a study coordinator:

Screening Information		
Weight	lbs:	kg:
Height	‘ “	m:
BMI (kg/m^2)		
Waist circumference (inches)		
Hip circumference (inches)		
Blood pressure	Systolic	Diastolic
Screening code (initials:mm:day:year; eg TR:07:22:10)		

Is subject fasted for blood sampling? YES NO

Do you have a nutrition study preference?

(a) Spice/Herb (free-living)
(b) Plant sterol cholesterol lowering (1 meal/day)
(c) Healthy oils (full-feeding)
(d) Barley fiber (full-feeding)

Appendix4. Consent form

UNIVERSITY OF MANITOBA

September 13, 2010

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study: **Efficacy of Plant Sterol-Fortified Dairy Product on Plasma Lipid and Plant Sterol Concentrations in Humans**

Investigator: Peter Jones, PhD

Richardson Centre for Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

Sponsor: Naturalis S.A

Avda. Pdte. Edo. Frei Montalva, 6000.
Quilicura Santiago, Chile
8700548
Phone: 56-2-4433522

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of study

The purpose of the study is to evaluate the lipid-lowering efficacy and safety of consumption of two plant sterol (PS) formulations compared to a placebo product. It has been shown that the ingestion of PS result in a favorable modification of lipid profiles. Therefore, it is anticipated that consumption of these PS enriched dairy products will improve lipid profile, as well as other health-related markers.

If you want to take part in this clinical study you will need to first do a genetic test. The purpose of this genetic test is to identify carriers of the genetic factor which leads to phytosterolemia. Phytosterolemia is a rare condition which causes a build-up of plant sterols in the body which can lead to heart disease. Not all carriers develop

phytosterolemia, but they may be at risk for cholesterol, heart valve and certain blood problems. Therefore, carriers of the gene need to be identified so they can receive necessary counselling or treatment. The genetic testing will require cheek swab samples from all participants.

Study procedures

You will be also asked to have a fasting (nothing to eat or drink 12 hours before the test) blood sample of approximately two teaspoons taken to measure your blood fat levels. If you meet eligibility requirements, you will be invited back for further screening where a fasting blood sample of four teaspoons will be taken to do a complete blood count, and biochemistry profile. We will also carry out a genetic test to identify if you have the disorder termed phytosterolemia. Phytosterolemia is a rare condition which causes a build-up of plant sterols in the body which can lead to heart disease. Therefore, individuals with phytosterolemia need to be identified and excluded from the study.

Prior to beginning the study, you will undergo a physical examination to determine whether you are eligible to participate. During the physical examination, the physician will measure your vital signs and ask you some questions regarding your medical history. An electrocardiogram (EKG) may be performed at the discretion of the physician in charge. Pregnancy tests will be performed for all pre-menopausal female subjects at screening visits and at the beginning of each phase, if it is positive at screening or during the study they will be asked to stop taking study treatment immediately and be withdrawn from the study. Any change in your health status at any point during the study needs to be reported to the study investigators.

The study will consist of 3 phases of 30 days during which you will consume your supper time meal (1 meal) at the centre along with your assigned treatment under supervision. You will follow your normal eating routine for the other 2 meals of the day. We ask you not to consume alcohol or caffeinated beverages throughout the phase. At the end of each phase, a washout period of 4 weeks will be followed during which you will consume your habitual diets. The 3 phases of treatments will include:

1) Study meal with placebo (regular dairy product).
2) Study meal with free PS dairy product (a novel formulation for dispersible free sterols in aqueous media produced by Harting SA company). The study treatment will provide 1.95g/d of PS.
3) Study meal with PS ester-fortified dairy product (a commercially available PS-ester preparation). The study treatment will provide 1.95g/d of PS.

This study is with a double –blinded design which means neither you nor the clinical staff will know which variation of the treatments you will be receiving. You will receive all 3 treatments, however, it will be unknown the order you will be given in what phase. In an emergency, this information will be made available.

During days 1, 2, 29 and 30 of each of the three phases of the trial, fasting blood samples (approximately 2 tablespoons will be taken on each blood draw day) will be obtained for assessment of blood fat levels and blood fat metabolism. Each blood test will take approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 18 tablespoons. The total blood volume required for this trial will be approximately 3.5 cups.

On day 1 and 30 of each phase you will be asked to give a urine sample. In addition, you will be asked to fill out a 3-day food diary at the end of each phase.

Risks and discomforts

In the event that you are identified as possessing phytosterolemia through the genetic test, this information could in the future affect your position if an insurance company or employer were to request it. We however, would never divulge such information without your written approval. All the results, including the genetic test result, will be kept confidential and will only be used for research purposes. The information will not be recorded in your hospital chart, should there be one.

There may be anxiety if in the future you are found as possessing phytosterolemia. Genetic counselling will always be available to you to discuss the result of the genetic test. .As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part.

The PS formulated with the proposed preparation procedures and at the proposed dose level, has been shown to have no known direct negative side effects on health in existing animal and human experiments. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site. In case you feel any discomfort during the experimental trial a physician, Dr. Kesselman, will be available to contact at any time. Dr. Kesselman can be reached at (204) 954-4486.

Benefits

You may not benefit from participation in this research; however, the study should contribute to a better understanding of the effect of PS formulations on health. It is also anticipated that oral intervention with these PS products will provide positive effects on lipid lowering efficacy and other health-related markers. In addition to the above, you will also receive your individual results and the average results of the entire group when they become available.

Costs

All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive. The study cost and honorariums will be covered by Naturalis S.A, the study sponsor.

Payment for participation

You will receive up to a maximum of $600 at completion of this study for your time and inconvenience of the study schedule. This amount will be divided into 3 equal portions and 1 portion given after each phase. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

Alternatives

You do not have to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study. You should be aware that medications exist as an alternative to treatment of lowering lipid and blood cholesterol levels.

Confidentiality

Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. With your permission, the study doctor will also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information. The Biomedical Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the RCFFN. Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, government organizations, insurance companies, or educational institutions. This restriction also applies to your spouse, other members of your family and your physician.

You will be assigned a subject code. The coding system of the study for subject identification will be the initials of each subject followed by a three-digit number. The three-digit number will be based on chronological order of subject selection. The identification codes corresponding to the study subjects will be on the written documents which will only be available to the RCFFN staff.

Study samples will be stored in the freezer at the RCFFN. Only the study coordinators and the principal investigator will have access to the samples. Your DNA samples used for genetic testing during screening and blood samples will not be used for any additional analyses, nor stored for any longer than 2 years, nor shared with any other group, other than is indicated in the protocol, without your specific consent.

Voluntary participation/withdrawal from the study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially close.

Medical care for injury related to the study

In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Kesselman at (204) 954-4486 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Kesselman will be contacted, who will inform you of the results.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

Investigator:	Dr. Peter Jones	Tel No.	204-474-9787
Study Physician	Dr. Edward Kesselman	Tel No.	204-954-4486

For questions about your rights as a research subject, you may contact:
The Biomedical Research Ethics Board, University of Manitoba at 789-3389
Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Consent

I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

Yes No

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.

2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.

4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

5. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.

6. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date/Time: _____

Printed name of above:_____

_____

For Clinical Study Coordinator: I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

Appendix 5: Sensory evaluation for flavored PS enriched-yogurt

UNIVERSITY OF MANITOBA

Date: Aug 26, 2010

Sensory Acceptance Test

Richardson Centre for Functional Foods and Nutraceuticals	**Title: Sensory Evaluation Report for Acceptance Testing of Flavored Plant Sterol Enriched- Yogurt**
	Prepared by: Mandana Amir Shaghaghi Approved by: Dr. Peter Jones

1. STUDY TITLE:

Efficacy of a Plant Sterol-Fortified Dairy Product on Plasma Lipid and Plant Sterol Concentrations in Humans

2. PURPOSE:

To determine if the flavored water dispersible sterol enriched- yogurt product (WD-PS-Yogurt) will be scored equal to or better than two other yogurt products (1. favored yogurt "without enhancement" (F-Yogurt) 2. Flavored sterol esters-enriched yogurt (PS-esters- Yogurt)) based on consumer acceptability of products through ranking evaluations @ 5% specific significance level.

3. MATERIALS AND METHODS:

Based on preliminary descriptive sensory test, 5 most favorite flavored yogurt samples (lemon, vanilla, grape, orange and apple) were prepared. A ranking test was used to determine the actual use (eating) of each flavored for WD-PS-Yogurt product (B.M. Watts et al, 1989).

Ten panelists from Richardson Center for Functional Foods and Nutraceuticals performed the evaluation (in-house panel). Three samples were presented for each flavored yogurt (100g yogurt +20 g WD-PS, 100g yogurt +3.37g PS-esters, and 100 g yogurt). All 3 treatments from each flavor were simultaneously presented to each assessor. The assessors were instructed to assign the most acceptable texture a rank value of (1), the sample with the next most acceptable texture a rank value of (2), and the sample with least acceptable texture a rank value of (3). Panelists were asked to not give the same rank to two samples.

3. RESULTS:

The ranked values assigned to each sample, for every favored yogurt, were tabulated (as shown in Tables 1-5). The samples were tested for significant differences by comparing the rank totals using the Friedman Test Tables (Appendix 1) (B.M. Watts et al, 1989 page 123).

3.1 Apple flavored yogurt

The ranked values given to each sample of apple flavored yogurts by all 10 panelists were shown in Table 1.

Based on the Friedman Test Table (Appendix 1), the tabulated critical value at p=0.005 for 10 panelists and three samples, is 11 (B.M. Watts et al, 1989 page 123).

Differences between rank total pairs (Table1):

C-A= 25-15= 10
C-B=25- 20=5 B-A= 20-15=5

Table 1: Tabulated Ranking for Acceptance Test Data for Apple Flavored yogurt

	Yogurt Varieties for each Flavor		
panelist	**A**	**B**	**C**
1	1	2	3
2	1	3	2
3	2	1	3
4	2	1	3
5	2	1	3
6	1	2	3
7	2	3	1
8	1	3	2
9	1	3	2
10	2	1	3
Rank Total	15	20	25

Note: Highest rank=1 (most acceptable texture)
Lowest rank=3 (least acceptable texture)

A: F- Yogurt
B: PS-ester-Yogurt
C: WD-PS-Yogurt

Based on the difference between rank total pairs, there were no significant different between the texture acceptances of PS-ester-Yogurt, F-Yogurt, and WD-PS- Yogurt.

3.2 Grape flavored yogurt

The ranked values given to each sample of Grape flavored yogurts by all 10 panelists were shown in Table 2.

Table 2: Tabulated Ranking for Acceptance Test Data for Grape Flavored yogurt

	Yogurt Varieties for each Flavor		
panelist	**A**	**B**	**C**
1	1	2	3
2	2	1	3
3	1	3	2
4	2	1	3
5	3	1	2
6	1	2	3
7	1	3	2
8	3	1	2
9	2	1	3
10	1	3	2
Rank Total	17	18	26

Note: Highest rank=1 (most acceptable texture), Lowest rank=3 (least acceptable texture)

Difference between rank total pairs:

C-A= 26-17= 9
C-B=26- 18= 8
A-B= 18- 17=1

There were no significant different between the texture acceptances of PS-ester-Yogurt, F-Yogurt, and WD-PS- Yogurt in the grape flavored yogurt.

3.3 Lemon flavored yogurt

The ranked values given to each sample of lemon flavored yogurts by all 10 panelists were shown in Table 3.

Table 3: Tabulated Ranking for Acceptance Test Data for Lemon Flavored yogurt

	Yogurt Varieties for each Flavor		
panelist	**A**	**B**	**C**
1	3	2	1
2	2	1	3
3	3	2	1
4	3	1	2
5	1	3	2
6	2	1	3
7	1	2	3
8	1	3	2
9	2	3	1
10	3	2	1
Rank Total	21	20	19

Note: Highest rank=1 (most acceptable texture)
Lowest rank=3 (least acceptable texture)

A: F- Yogurt
B: PS-ester-Yogurt
C: WD-PS-Yogurt

Differences between rank total pairs:

C-A= 19-21= -2
C-B=19-20= -1
B-A= 20-21= -1

No significant different between the texture acceptances of PS-ester-Y and F-Yogurt and WD-PS-Yogurt and F-Yogurt has been found.

3.4 Orange flavored yogurt

The ranked values given to each sample of orange flavored yogurts by all 10 panelists were shown in Table 4.

Differences between rank total pairs:

C-A= 25-17= 8
C-B=25- 18=7
B-A= 18-17=1

Table 4: Tabulated Ranking for Acceptance Test Data for Orange Flavored Yogurt

	Yogurt Varieties for each Flavor		
panelist	**A**	**B**	**C**
1	1	2	3
2	2	1	3
3	3	1	2
4	2	1	3
5	1	2	3
6	2	1	3
7	2	1	3
8	1	3	2
9	1	3	2
10	2	3	1
Rank Total	17	18	25

Note: Highest rank=1 (most acceptable texture) A: F- Yogurt
Lowest rank=3 (least acceptable texture) B: PS-ester-Yogurt
C: WD-PS-Yogurt

No significant different between the texture acceptances of PS-ester-Y and F-Yogurt and WD-PS-Yogurt and F-Yogurt has been found.

3.5 Vanilla flavored yogurt

The ranked values given to each sample of Vanilla flavored yogurts by all 10 panelists were shown in Table 5.

Differences between rank total pairs:
C-A= 22-23= -1
C-B=22-25 =-3
B-A= 25-23=2

Table 5: Tabulated Ranking for Acceptance Test Data for Vanilla Flavored yogurt

	Yogurt Varieties for each Flavor		
panelist	**A**	**B**	**C**
1	1	2	3
2	1	3	2
3	1	3	2
4	3	2	1
5	1	3	2
6	1	2	3
7	2	1	3
8	1	2	3
9	1	3	2
10	2	3	1
Rank Total	24	24	22

Note: Highest rank=1 (most acceptable texture) A: F- Yogurt
Lowest rank=3 (least acceptable texture) B: PS-ester-Yogurt
C: WD-PS-Yogurt

There were no significant different between the texture acceptances of PS-ester-Yogurt, F-Yogurt, and WD-PS- Yogurt in the grape flavored yogurt. Interestingly, vanilla flavored WD-PS-Yogurt had the most preferable texture among the three.

4. CONCLUSION:

In general, the in-house panel found the texture of flavored WD-PS-Yogurt and PS-ester-Yogurt less acceptable than the texture of F-Yogurt. However, no significant difference has been found. It appears that all 5 flavors (lemon, vanilla, grape, orange and apple) can be included in the making plant sterol enriched-yogurt for the purpose of increasing variety, regardless of the formulation of Plant sterol (WD-PS/PS-ester sterol).

Reference:

Watts, B.M., Ylimaki, L. E., Jeffery, L.E., Elias, L.G., (1989). Basic Sensory methods for food evaluation: Ottawa, Ont, IDRC.

Appendix 6: Standard operation procedure (SOP1)

UNIVERSITY OF MANITOBA

Date: Sept 10, 2010

Standard Operation Procedure

Richardson Centre for Functional Foods and Nutraceuticals	**Title: Mixing Yogurt with Water Dispersible Sterol (WD-PS)**	
	Version: H	**SOP Number: YOG- NDS-02**

STUDY TITLE

Efficacy of a Plant Sterol-Fortified Dairy Product on Plasma Lipid and Plant Sterol Concentrations in Humans

1. PURPOSE

1.1 This Standard Operation Procedure (SOP) will detail the process of preparing flavored yogurt and mixing it with WD-PS.

2. SCOPE

2.1 This procedure applies to all RCFFN metabolic kitchen personnel responsible for preparing favored yogurt mixed with WD-PS.

3. RESPONSIBILITIES

3.1 Graduate student/ Clinical coordinators are responsible for training the kitchen staff.
3.2 Graduate student is responsible for Quality Control Check for adequacy of plant sterol in yogurt (2 g in 100 yogurt)
3.3 Graduate student is responsible for updating this SOP, if necessary.

4. REFERENCES (NOT APPLICABLE)

5. DEFINITIONS (NOT APPLICABLE)

6. EQUIPMENT

6.1 Hobart mixer HL600
6.2 Digital scale

7. MATERIALS

7.1 Pasteurized plain yogurt 4% MF (Natures Treat, Dairyland, Canada)
7.2 NDS 10%
7.3 Oil 'flavor' (LORANN GOURMET)
7.4 Food colour (CLUB HOUSE)
7.5 Sweetener (SPLENDA)

8. PROCEDURE

To make 12 kg flavored plant sterol-enriched yogurt (2g plant sterol in 100 g yogurt):

8.1 Pour 10 kg of yogurt in the sterile mixer bowl
8.2 Add 2 kg WD-PS
8.3 Add 80 g sweetener
8.4 Add 1 teaspoon (5 ml) flavor *
8.5 Add 1.5 teaspoon (6.6 ml) colour **
8.5 Mix for 3 minutes on the 1st speed
8.6 Keep refrigerated for maximum two weeks

9. ATTACHMENTS (NOT APPLICABLE)

10. FORMS (NOT APPLICABLE)

---

* Approximately 1 drop of flavor for 100g yogurt
** Approximately 1.5 drops of colour for 100g yogurt

11. REVISION HISTORY

Version	Change Description	Date Adopted
H	updated SOP format	7 Sept, 2010
F	sensory test 2	26 Aug, 2010
E	quality control check for adequacy of plant sterol in yogurt (2 g/ 100 yogurt)	6 Aug, 2010
D	minor changes on the amount of sweetener and flavor based on the result of the test	2 Aug, 2010
C	sensory test 1	30 July, 2010
B	method established	27 July, 2010
A	document initiated	26 July, 2010

12. APPROVALS

Prepared by: Mandana Amir Shaghaghi Date: 8 September 2010

Approved by: Dr. Peter Jones Date: 9 September 2010

Appendix7: Standard operation procedure (SOP2)

UNIVERSITY OF MANITOBA

Date: Sep 10, 2010

Standard Operation Procedure

Richardson Centre for Functional Foods and Nutraceuticals	**Title: Mixing Yogurt with plant sterol ester (PS-ester)**	
	Version: H	**SOP Number: YOG- PSE-01**

STUDY TITLE

Efficacy of a Plant Sterol-Fortified Dairy Product on Plasma Lipid and Plant Sterol Concentrations in Humans

1. PURPOSE

1.1 This Standard Operation Procedure (SOP) will detail the process of preparing flavored yogurt and mixing it with PSE.

2. SCOPE

2.1 This procedure applies to all RCFFN metabolic kitchen personnel responsible for preparing favored yogurt mixed with PS-ester.

3. RESPONSIBILITIES

3.1 Graduate student/ Clinical coordinators are responsible for training the kitchen staff.
3.2 Graduate student is responsible for Quality Control Check for adequacy of plant sterol in yogurt (2 g in 100 yogurt)
3.3 Graduate student is responsible for updating this SOP, if necessary.

4. REFERENCES (NOT APPLICABLE)

5. DEFINITIONS (NOT APPLICABLE)

6. EQUIPMENT

6.1 Water bath VWR1227
6.2 Hobart mixer HL600
6.3 Digital scale

7. MATERIALS

7.1 Pasteurized plain yogurt 4% MF (Natures Treat, Dairyland, Canada)
7.2 PSE
7.3 Oil 'flavor' (LORANN GOURMET)
7.4 Food colour (CLUB HOUSE)
7.5 Sweetener (SPLENDA)

8. PROCEDURE

To make 10.328 kg flavored plant sterol-enriched yogurt (2g plant sterol in 100 g):

8.1 Melt 337 PSE at 60°C in the water bath (3 minutes)
8.2 Pour 10 kg of yogurt in the sterile mixer bowl
8.3 Add melted PS-ester
8.4 Add 80 g sweetener
8.5 Add 1 teaspoon (5 ml) flavor *
8.6 Add 1.5 teaspoon (6.6 ml) colour **
8.7 Mix for 3 minutes on the 1st speed
8.8 Keep refrigerated for maximum two weeks

9. ATTACHMENTS (NOT APPLICABLE)

10. FORMS (NOT APPLICABLE)

---

* Approximately 1 drop of flavor for 100g yogurt
** Approximately 1.5 drops of colour for 100g yogurt

11. REVISION HISTORY

Version	Change Description	Date Adopted
H	updated SOP format	7 Sept, 2010
F	sensory test 2	26 Aug, 2010
E	quality control check for adequacy of plant sterol in yogurt (2 g/ 100 yogurt)	6 Aug, 2010
D	minor changes on the amount of sweetener and flavor based on the result of the test	2 Aug, 2010
C	sensory test 1	30 July, 2010
B	method established	27 July, 2010
A	document initiated	26 July, 2010

12. APPROVALS

Prepared by: Mandana Amir Shaghaghi Date: 8 September, 2010

Approved by: Dr. Peter Jones Date: 9 September, 2010